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The Tim family of molecules in the chicken – important differences from mammals

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Author's declaration

I declare that the work in this dissertation is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

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Abstract

T cell immunoglobulin and mucin (Tim) family molecules are cell membrane proteins with four functional Tim family members in mouse, and three in human. They are preferentially expressed on immune cells with Tim1 on Th2 cells, Tim3 on Th1 cells and Tim4 on antigen-presenting cells (APCs). They have several roles, including regulating immune responses and mediating phagocytosis of dead cells. However, little is known about them beyond these two species, and nothing outside mammals. To investigate the Tim family in the chicken, the genes were identified and cDNAs cloned. Differently to mammals, the chicken genome only contains genes for Tim1 and Tim4. Chicken Tim1 (chTim1) has similar mRNA expression patterns to those of mammalian Tim1 in lymphoid tissues and immune cells. Interestingly, chTim4 has at least four splice variants – an extra short isoform (chTimeS) lacking exons 5, 6, 7 and 8, a short isoform (chTim4S) without exons 3, 4 and 5, a long isoform (chTim4L) with all exons and an extra long isoform (chTim4eL), which is similar to chTim4L but with a longer exon 3. The chTim4S is a homologue of mammalian Tim4 with constitutive expression in lymphoid tissues and immune cells; other chTim4 variants showed inducible or cell-specific expression patterns. Like mammalian Tim4, chTim4S is expressed by APCs; but differently to mammals, chTim4S is also expressed by γδ T cells, suggesting a unique role for chTim4 in this population of T cells.

The biological activities of the chicken Tim family molecules were also investigated using chTim-Ig fusion proteins. Like mammals, chTim1 and chTim4S fusion proteins can specifically recognise phosphatidylserine (PS), an indicator of apoptotic cells, suggesting they are PS receptors. Pre-incubation with PS blocked binding of the chTim4S fusion protein to PS-exposing apoptotic cells. Physiologically, recognition of PS by the chTim proteins mediates apoptotic cell clearance, which was demonstrated using chTim-transfected fibroblast cells (3T3), which significantly increased their uptake of apoptotic cells compared with untransfected cells. The chTim4-Ig fusion protein also had costimulatory activity on chicken T cells.

Monoclonal antibodies against the chTim proteins were generated. They specifically recognise their own antigen tested intensively by different immunological assays. ChTim4L is expressed intracellularly in freshly-isolated splenocytes rather than on the surface, whereas PMA-stimulated splenocytes express chTim4S and chTim4L on the cell surface. Like mammals, chicken splenic macrophages also express chTim4S and chTim4L. Both of them are also expressed by bone marrow-derived macrophages but not bone marrow-derived DCs. The chTim1 protein was detected at high levels in bursal cells and splenocytes by western blot analysis using polyclonal anti-chTim1 serum, which is consistent with its mRNA expression pattern through qRT-PCR analysis, suggesting B and T cells may express chTim1, consistent with its expression in mammals. Mammalian Tim1 is expressed on Th2 cells, its ligand, Tim4, on APCs; the interaction between them drives Th2 cell proliferation. The knowledge from this study will help to further dissect how the chicken's Th2 responses are regulated through cell surface molecules.

List of contents

List of tables

List of figures

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Abbreviations and acronyms

%	Percentage
α	Alpha
Ab(s)	Antibod(ies)
AEC	3-amino-9-ethylcarbazole
APC	Antigen presenting cells
APS	Ammonium persulfate solution
β	Beta
BSA	Bovine serum albumin
bp	Base pairs
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
ch	Chicken
cm	Centimetre
ConA	Concanavalin A
CpG	Unmethylated CpG synthetic oligonucleotides
CS	Chicken serum
δ	Delta
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
γ	Gamma
g	Gram
g	Relative Centrifugation Force
h	Hour
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus

HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kd	Kilobase
kDa	KiloDalton
l	Litre
LB	Luria-Bertani
LPS	Lipopolysaccharide
M	Molar
MDV	Marek's disease virus
mg	Milligram
mM	Millimolar
$MgCl_2$	Magnesium sulphate
MHC	Major histocompatibility complex
ml	Microlitre
µg	Microgram
µl	Microlitre
ng	Nanogram
nm	Nanometre
NH_2	Amino
ºC	Degree(s) Celsius
OPD	o-Phenylenediamine dihydrochloride
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cell
PEG	Polyethylene glycol
PI3K	Phosphatidylinositol 3-kinase
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
TBE	Tris/Borate/EDTA buffer
Taq	Thermus aquaticus
TEMED	N,N,N',N'-tetramethylethylenediamine
v/v	Volume per volume
w/v	Weight per volume

Chapter 1. General Introduction.

1.1 The chicken

The origin of the domestic chicken has been under debate for centuries, the most common theory being that modern domestic chickens are descendents of the Southeast Asian Jungle Fowl (Eriksson et al., 2008). Huge achievements in chicken production have been made by the few world-leading chicken breeders. For example, constant genetic selection and improvement in nutrition have led to very fast body growth rate in modern broiler strains. Nowadays, a broiler chicken only needs about 40 days to reach a target 2 kg body weight. Moreover, high rates of egg production by layers have also been selected based on genetic traits; selection of a growth hormone gene allele resulting in a delayed onset of ovulation led to an overall increase of egg production by 15% (age at first egg to 497 days) in white leghorn chickens (Kuhnlein et al., 1997). As well as progress in chicken breeding, other improvements in the poultry industry, such as nutrition and the industrial processing of chicken food, intensive rearing techniques, disease control programmes, etc. have resulted in a sustainable and massive output of chicken production, putting poultry production central to the food security agenda. Chicken meat consumption has increased to around 91 million tonnes in 2012. On a per-person basis, average poultry meat consumption on a global basis has continued to rise from 11.1 kg in 2000 to around 14.6 kg in 2012 (http://www.thepoultry site.com/articles/2640/global-poultry-trends-2012). Chicken meat is the most frequently eaten meat (>40%) in the UK, where consumption has doubled in the past 20 years, resulting in the UK producing 850 million chickens per year (Kaiser, 2010). Additionally, the

environmental impacts of meat production have become a factor that affects policy making. The production of chicken products has much lower greenhouse-gas emissions (McMichael et al., 2007) and water usage (Hoekstra et al., 2003) than the other two major meat products, pork and beef. To balance the environmental impact of increased meat supplies, increasing chicken production will become the primary option in the future. In developing countries, especially in their poor rural areas, eggs from free-range birds become an affordable daily protein source to resolve the problem of child malnutrition.

Efficient poultry production requires a rapid expansion of the poultry production chain, and poultry health and welfare are also vital to the supply and safety of food, which can also significantly influence economic and societal prosperity. For example, salmonellosis does not generally cause severe symptoms in poultry, but the eggs and meat of infected animals can become a reservoir of infection for the human consumer. Legislation discontinuing the use of antibiotics and other drugs increases the risks to the poultry industry of pathogens, such as bacterial and parasitic infections. Virus infections also persistently threaten the poultry industry, such as Marek's disease. In spite of the success of vaccines in reducing losses from the disease in the last 30 years, the use of these vaccines is thought to have driven the virus to evolve to higher virulence to overcome the immune responses induced by the vaccines (Nair et al., 2005). Highly pathogenic avian influenza virus (AIV), for example H5N1, can cause mortality in chickens as high as 100% and can also be fatal to humans (Peiris et al., 2007). Even a lowly pathogenic avian influenza, H7N9, can become a significant pandemic threat to public health (Gao et al., 2013). Migratory waterfowl are a carrier for highly

pathogenic AIV and promote virus transmission across continents (Liu et al., 2005). Increasing zoonotic threats, especially currently from avian influenza, necessitate the search for alternative control methods. These must be based on the development of detailed knowledge of the mechanisms that the host uses to resolve infection with pathogens and that vaccination, genetics or other means can manipulate the capability of host to resist pathogen invasion.

1.2 Overview of the human immune system

The human immune system is crucial in protecting the host from pathogen infection. This task is fulfilled by a well-organised system, composed of different organs that are networked with lymphatic vessels, in which immune cells are collected from peripheral tissues and pass through the lymph nodes before being put back into the blood. The bone marrow and the thymus are the primary lymphoid organs (also called central lymphoid organs); the former is for the generation of primitive immune cells and the maturation of B cells, and the latter for T cell maturation (Schultz et al., 1987). The secondary lymphoid structures, also called peripheral lymphoid organs, include the spleen, lymph nodes, tonsils, Peyer's patches and diffuse lymphoid aggregates in mucous membranes, such as gut- and lung-associated lymphoid tissues, to which immune cells are homed, become activated and cross-talk to each other (Schultz et al., 1987).

Two systems are used to defend the host's body against foreign invaders. A non-specific defence system relies on the body's natural ability to form physical barriers to prevent foreign matter entry, such as skin and mucous membranes and fluids, e.g. tears and saliva. However, when pathogens break through the physical

defence barriers and enter the body, another defence system, the immune system, is then initiated to combat the threatening invader. The major function of the immune system is to protect the host from environmental agents such as microbes or chemicals, thereby preserving the integrity of the body. This is done by the recognition of self and response to non-self (Beutler et al., 2004; Janeway et al., 2002). The immune response has been somewhat artificially divided into innate and adaptive immunity.

1.2.1 The human innate immune response

The human innate immune system is a universal form of host defence against infection, and the innate immune response is not altered upon repeat exposure to the same pathogen. The innate immune response requires immune cells, as shown in Figure 1.1, including mast cells, basophils, eosinophils and phagocytic cells, such as macrophages, neutrophils and dendritic cells (DCs). Natural killer (NK) and γδ T cells are involved in not only innate immunity but also adaptive immune responses. These cells express soluble and cell-associated germline-encoded pattern recognition receptors (PRRs), which are already encoded in the genome without any DNA rearrangement. Innate immune cells can respond to pathogens through the recognition by PRRs of pathogen-associated molecular patterns (PAMPs), which are evolutionarily conserved microbial components present in pathogens, such as cell wall components, flagella, lipoproteins, and nucleic acids of bacterial, fungal, and viral origin (Janeway et al., 2002; Takeuchi et al., 2010). The complement system is a large number of distinct plasma proteins that react with one another to opsonize pathogens and induce a series of inflammatory responses that help innate cells to fight infection.

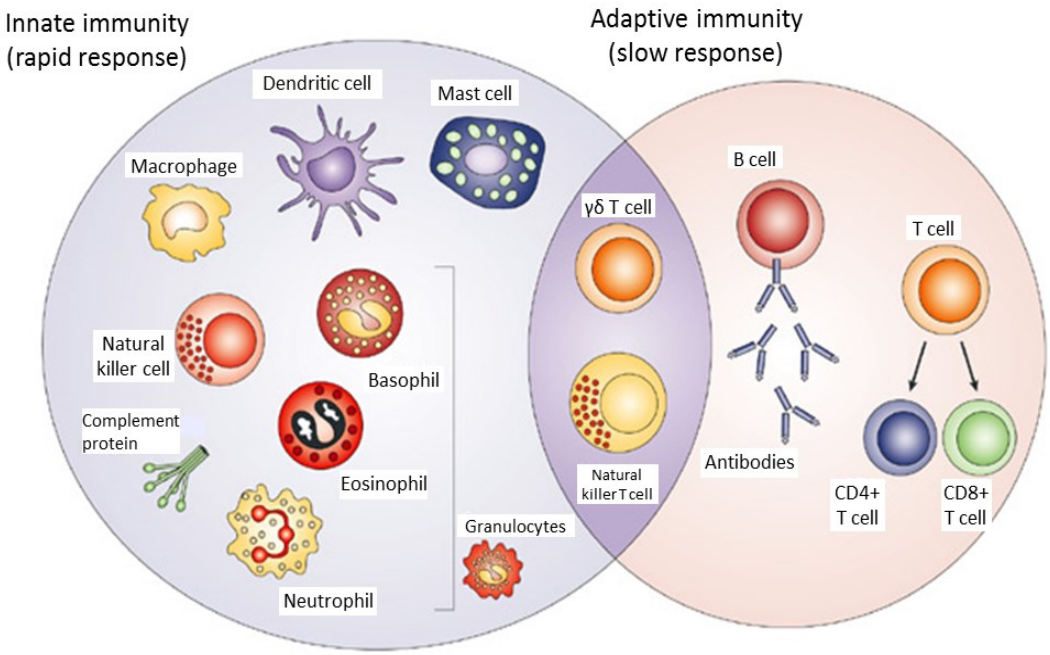


Figure 1.1. Innate and adaptive immune responses. The innate immune response functions as the first line of defence against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells and $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity (modified from Dranoff, 2004).

Based on their molecular structure, PRRs can be classified into several families, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and RIG-I-like receptors (RLRs) (O'Neill and Bowie, 2010). The TLRs recognise a remarkably broad spectrum of PAMPs expressed by viral, bacterial, fungal, and parasitic pathogens. In human, as shown in Figure 1.2, TLR1, TLR2, TLR4, TLR5 and TLR6 are primarily expressed on the plasma membrane, where they sense specific molecules on the surface of microbes. In contrast, TLR3, TLR7 and TLR9 traffic from the endoplasmic reticulum to endolysosomal compartments, where they recognize RNA and DNA derived from pathogens (Macagno et al., 2007; Nowarski et al., 2013). TLR8 was initially thought to be non-functional in mice; however, human TLR8 can sense bacterial RNA released within phagosomal vacuoles, as do other endosomal TLRs (Davila et al., 2008; Gantier et al., 2010). TLR10 is the only family member without a defined agonist or function (Guan et al., 2010). CLRs include at least 17 transmembrane proteins, which are either membrane-bound or secreted, binding to carbohydrate moieties such as mannose, fucose, and β-glucan on pathogens (Takeuchi et al., 2010; Osorio et al., 2011). Differently to the TLRs and CLRs, the NLRs and RLRs lack transmembrane domains and function as cytoplasmic sensors for intracellular pathogen invasion. RLRs are cytoplasmic helicases that sense viral single- or double-stranded RNA as non-self and bind to them through recognition of the PAMP motifs within viral RNAs (Ramos et al., 2011). More than 20 NLRs are predicted in the human genome. All NLRs contain a NH_2-terminal nucleotide-binding oligomerization domain that mediates the formation of self oligomers, and a carboxy-terminal leucine-rich repeat (LRR),

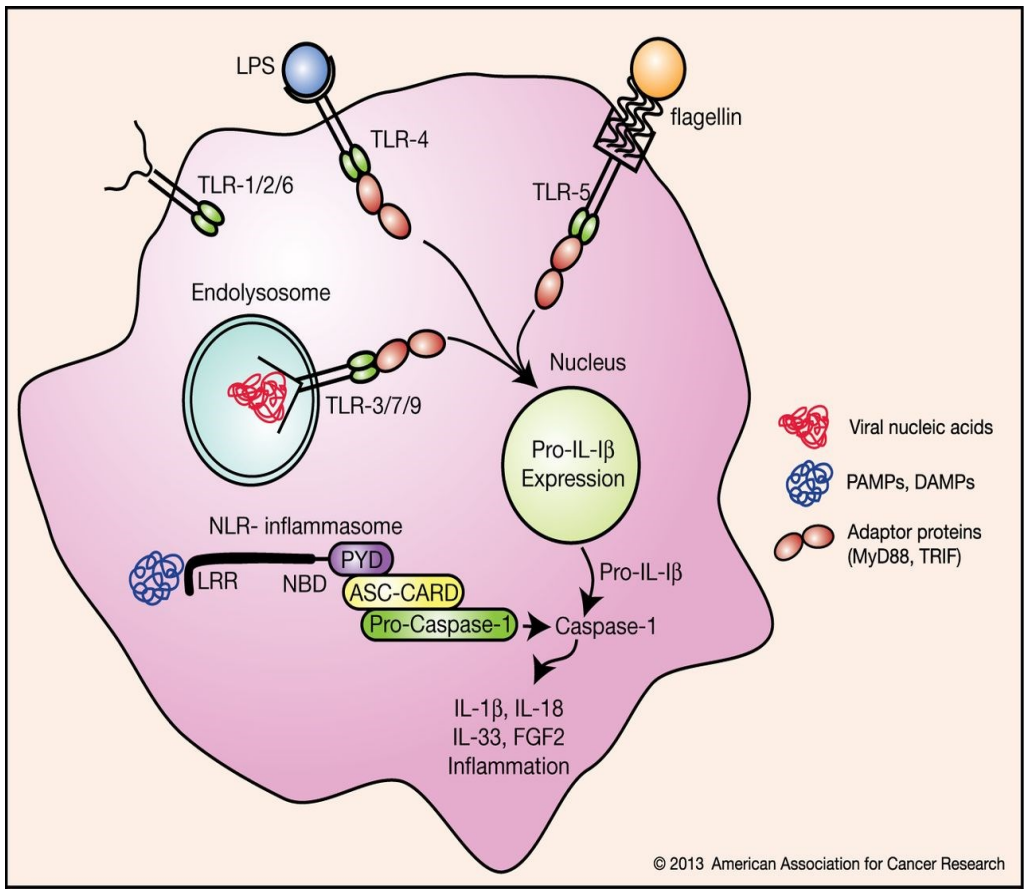


Figure 1.2. TLRs and NLRs involved in human innate immune response. TLRs, expressed on the cell surface or intracellular vesicular membranes of innate immune cells and epithelial cells, sense and capture danger signals. Cell surface TLRs, including TLR1, 2, 4, 5 and 6, sense exogenous pathogens through recognition of PAMPs on their surface and TLRs, including TLR3, 7 and 9, expressed on endolysosomal compartments recognize endogenous ligands, such as RNA and DNA derived from pathogens. NLRs are cytosolic PRRs. Sensing endogenous PAMPs through their leucine-rich repeat domain (LRR) trigger a cascade process to assemble an inflammasome, which in turn promotes the maturation of bioactive caspase-1. Activated caspase-1 has proteolytical activity on pro-IL-1β and IL-18, whose expression is driven by TLR signalling, converting them into mature forms, and increased IL-33 and fibroblast growth factor-2 (FGF-2) production (Nowarski et al., 2013).

which is responsible for detection of PAMPs (Elinav et al., 2011). As shown in Figure 1.2, TLR signalling triggers pro-IL-1β synthesis, which requires a proteolytical activation by a ternary protein complex, called an inflammasome, to become the mature form of IL-1β. Sensing of PAMPs leads to NLRs assembling into inflammasomes. NLRs are composed of a LRR domain, a central nucleotide-binding domain (NBD), and an NH_2-terminal pyrin domain (PYD). The LRR and NBD modules are involved in ligand sensing and autoregulation, respectively. PYD is usually involved in the recruitment of the adaptor protein apoptosis-associated speck-like protein (ASC) containing a caspase-associated and recruitment domain (CARD), which in turn is associated with pro-caspase-1. Activation of the inflammasome complex leads to autocleavage and activation of pro-caspase-1, which in turn is responsible for processing and secretion of the mature forms of the pro-inflammatory cytokines IL-1β and IL-18, and the secretion of IL-33 and fibroblast growth factor-2 (FGF-2) (Nowarski et al., 2013).

Macrophages are important phagocytes, the latter also including neutrophils, in the innate immune system and are widely present in peripheral tissues. DCs are also phagocytes, but don't have killing capacity of pathogen; their role in innate immune response is to phagocytose foreign antigens derived from pathogens for presenting them to T cells (Hashimoto et al., 2011). Pathogen invasion can cause inflammatory responses at infection sites, which are initiated by phagocytic processing of pathogens by macrophages. The pathogens are engulfed by macrophages through PRRs and destroyed in phagolysosomes by acid and enzymes (Janeway et al., 2002). Phagocytosed pathogens also stimulate the macrophage to produce cytokines and chemokines, thereby attracting other cells to the infection site

and promoting inflammation. The inflammatory response in infected tissue causes vasodilation of the blood vessels, facilitating access of phagocytes, especially neutrophils, to the pathogens. The mast cells, basophils and eosinophils at the infection site are also involved in the inflammation. When these cells are activated at the infectious site, they produce histamines and chemokines; the former dilate blood vessels, causing the leakage of immune cells into tissues, and the latter then direct these cells, especially neutrophils and macrophages, through a chemotaxis process, to move towards infection sites (Janeway et al., 2002; Takeuchi et al., 2010). NK cells also are a component of the innate immune system which does not directly attack invading microbes. However, they destroy pathogen-infected host cells to release pathogen components, facilitating pathogen clearance by phagocytes (Vivier, 2006).

Pathogen-activated macrophages produce cytokines, including the pro-inflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α, which promote and control early inflammatory responses, and type I interferons, IFN-α and IFN-β, to primarily block viral replication. PAMPs also induce DC maturation with upregulation of most of the requisite molecules, including costimulatory molecules, MHC class II, and CCR7 surface expression, to prime effectively the adaptive response by presenting antigen to T cells (Janeway et al., 2002, Macagno et al., 2007).

1.2.2 The human adaptive immune response

When pathogens overwhelm the innate immune responses and are persistently present in the host, a more effective and specific immune response, called the adaptive immune response, is triggered, consisting of antibody responses, also called humoral immunity, and cell-mediated responses, carried out by B cells

and T cells (Zielinski et al., 2011). B cells are involved in humoral immune responses through creating antigen-specific antibodies that circulate in the host to bind specifically to foreign antigens. Binding of antibody can inhibit virus replication, inactivate viral and microbial toxins and initiate other immune cells to attack the pathogens (Sallusto et al., 2010). T cells are involved in cell-mediated immunity, where activated T cells release various cytokines to enhance other cell activities, including macrophages, natural killer cells (NK), antigen-specific cytotoxic T lymphocytes, as well as B cells (Sallusto et al., 2010). A key feature of the adaptive immune response is to develop immunological memory to a pathogen, whereby when the same pathogen invades, the host can rapidly recall the previous response to battle the infection (Sallusto et al., 2010; Zielinski et al., 2011).

B cell development in human starts in the bone marrow with the commitment of haematopoietic stem cells to the B cell lineage. The B cell progenitors sequentially express and assemble the components of the B cell antigen receptor (BCR), including immunoglobulin (Ig) heavy and light chains as well as the signalling proteins Igα and Igβ, resulting in immature B cells with surface-bound IgM differentiated in the bone marrow. These immature B cells migrate to secondary lymphoid tissues (such as the spleen, lymph nodes, Peyer's patches, etc.) where they are called transitional B cells. Immature B cells, which have never been exposed to an antigen, are known as naïve B cells and express only the IgM isotype on a cell surface in a membrane-bound form. When B cells begin to express both membrane-bound IgM and IgD, they reach maturity and are called mature B cells. These B cells are ready to respond to antigen (Hardy et al., 2000).

Mature B cells are activated by antigens from pathogens through cell surface-bound IgM or IgD in a T cell-independent manner or through the CD40-CD40L pathway and cytokines in a T cell-dependent manner (Lanzavecchia et al., 2002). Activated B cells divide and differentiate into antibody-producing cells, called plasma cells, that produce secreted antibody, including IgE, IgA or IgG, or memory cells that survive for years afterwards to allow the immune system to remember and rapidly respond to the same antigen upon future exposure. An antibody is a large Y-shaped protein, consisting of two heavy chains and two light chains. Antibody can specifically recognise foreign antigens from bacteria and virus through its variable domain (Manz et al., 2005). However, microbes are highly diverse, thus successful recognition and eradication of them requires a high diversity of antibodies. Three ways are used to produce a sufficient number of different antibodies by the host. The variable domain of the Ig heavy chain is encoded by several pieces, called variable (V), diversity (D) and joining (J) genes, each of these genes having multiple segments. Somatic recombination of segments from the V, D and J genes, also known as V(D)J recombination, results in a variety of Ig heavy chains (Figure 1.3). The light chain of Ig has similar recombination between variable (V_L), joining (J_L) and constant (C_L) gene segments, resulting in a variety of light chains. The different combinations of heavy and light chains also introduce more diversity. Another way is that the genes encoding the variable domains undergo a high rate of point mutation, by a process called somatic hypermutation (SHM), during expansion of activated B cells upon antigen stimulation, also allowing a vast antibody repertoire to be generated (Manz et al., 2005; Corcoran, 2010).

T lymphocyte progenitors arise in the bone marrow and migrate via the blood

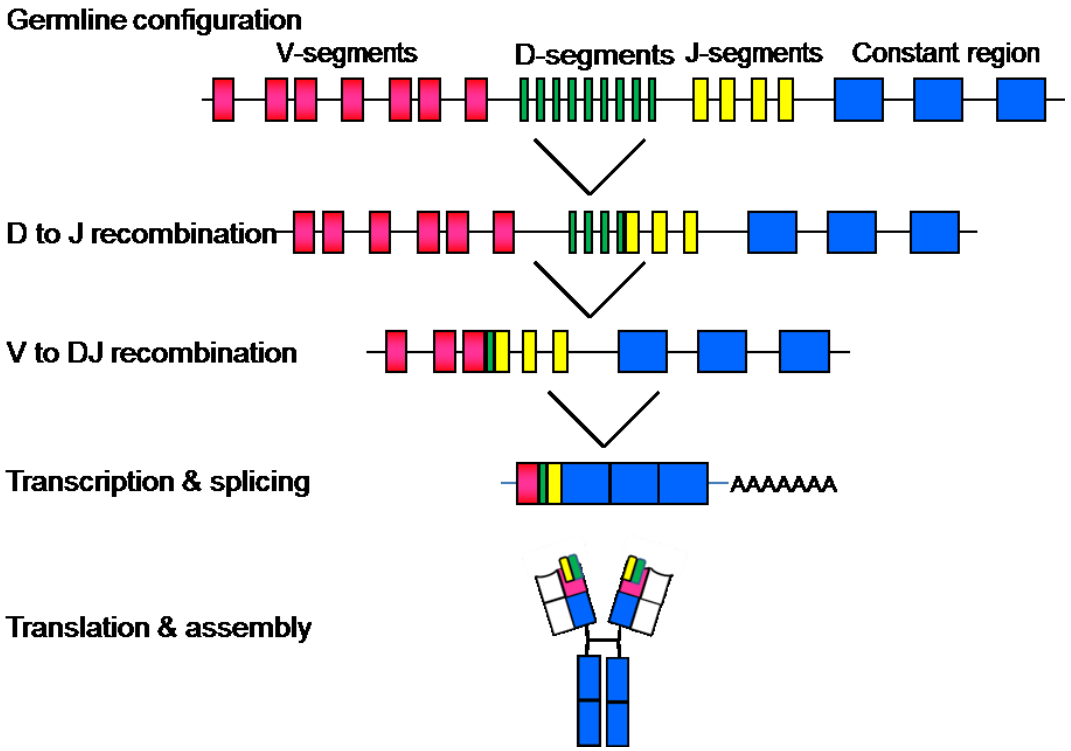


Figure 1.3. Schematic diagram showing somatic recombination of the Ig heavy chain. The Ig heavy chain is encoded by several genes, including a gene encoding the constant region and variable (V), diversity (D) and joining (J) genes encoding the variable domain. V(D)J recombination of their multiple segments results in a large variety of Ig heavy chain variable domain to recognise a diversity of antigens. The two heavy chains are assembled with two Ig light chains (shown in white boxes) to form a fully functional antibody (modified from Corcoran, 2010).

and a chemotaxis process to the thymus. These early T cell progenitors (ETP) neither express CD4 nor CD8, and are also called double negative (DN). At an early stage of T lineage development, these ETPs experience differential expression of cell surface CD markers and rearrangement of T cell receptor (TCR) genes in the environment of thymus, resulting in two populations: γδ T cells with $CD3^-$ $CD44^-$ $CD25^+$ and αβ T cell progenitors with $CD3^+$ $CD44^-$ $CD25^+$. This stage of differentiation within the thymus requires intimate contact with the epithelial stromal cells, which express notch ligands, essential growth factors (IL-7) and morphogens (sonic hedgehog proteins) important for T cell development (Shortman et al., 1996; Koch et al., 2011). When αβ T cell progenitors lose their dependence on Notch ligand, IL-7 and sonic hedgehog, the cells then differentiate into $CD4^+$ $CD8^+$ double positive cells (DP). The cells that are not able to generate a functional TCR will die through apoptosis. Those DP cells with a functional TCR are then exposed to endogenous peptides presented by MHC molecules expressed on thymic cells, resulting in positive and negative selection. In positive selection, TCR interaction with a self peptide-MHC complex leads DP cells to lose one of the co-receptor molecules (CD4 or CD8), also resulting in a self MHC-restricted T cell repertoire. The "single-positive" (SP) T cells ($CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$) then undergo negative selection by affinity with self peptide-MHC. Those cells strongly bound to an MHC complex will die. Following selection, surviving αβ T cells migrate from the thymus to secondary lymphoid organs (Gameiro et al., 2010; Koch et al., 2011).

Dendritic cells (DCs) are antigen-presenting cells crucial for bridging the innate and adaptive immune response to infection. In human, DC subtypes include type I IFN-producing plasmacytoid DCs (pDCs) and conventional DCs (cDCs).

pDCs are innate immune cells present in the blood and peripheral lymphoid organs with a different cell surface phenotype to cDCs. They play crucial roles in anti-viral infection by producing large amounts of IFN-α and IFN-β (McKenna et al., 2005; Tversky et al., 2008). cDCs are widely present in lymphoid tissues, such as the thymus, spleen and lymph nodes, and non-lymphoid tissues, such as skin and mucosal-associated tissues. They are crucial for presenting foreign antigen to T cells (Wu et al., 2007). Immature cDCs differentiate from haematopoietic bone marrow progenitor cells. Once they recognise foreign antigens derived from pathogens by surface PRRs, the pathogenic proteins are then internalised and degraded into small pieces, which can be presented on the DC cell surface in the context of MHC molecules. Simultaneously, costimulatory molecules, such as CD80, CD86 and CD40, are upregulated on the DC surface. Mature DCs also upregulate expression of CCR7, a chemotactic receptor that guides trafficking of the DCs to the spleen or lymph nodes, where DCs then present foreign antigens to T cells, and stimulate T cell proliferation along with costimulatory molecules (Suciu-Foca et al., 2005). MHC molecules are highly polymorphic to be suitable for presenting diverse peptides. They can be divided into class I and class II. MHC class I molecules are expressed by nearly every nucleated cell, whereas MHC class II molecules are normally expressed on antigen-presenting cells (APCs), including DCs, macrophages and B cells (Martinsohn et al., 1999).

As described before, after T cell lineage development in the thymus, they mainly become single positive $CD8^+$ or $CD4^+$ T cells. After activation by antigen-armed DCs, naïve T cells can rapidly expand and differentiate into effector T cells to cope with the infection. Most of the effector cells will die when the host recovers

from the infection, but a few of these cells are retained as memory cells to mount an effective response when reencountering the same antigen in future. $CD8^+$ T cells normally are cytotoxic T lymphocytes (CTL) and activated through peptide-loaded MHC class I molecules. The effector CTL can induce the death of cells infected with viruses or other pathogens (Harty et al., 2000). $CD4^+$ T cells are helper T (Th) cells, which are immune response mediators and play roles in directing other immune cells to establish and maximise the capabilities of the acquired immune response. APCs present foreign antigen through MHC class II to naive $CD4^+$ T cells. Activated $CD4^+$ T cells further differentiate into different effector T helper subsets, as shown in Figure 1.4, including Th1, Th2, Th17, Th9, Th22, T_{reg} and T_{fh} (Zielinski et al., 2011). The Th1/Th2 paradigm is the best-studied model for T helper subset differentiation, and will be specifically described later. Th17 cells produce IL-17 as their signature cytokine and specifically express a transcpritional factor, RORγt, which regulates Th17 cell differentiation. Th17 cells play potent roles in the elimination of fungi and extracellular bacteria (Basu et al., 2013). Th22 cells are a recently characterised subset, based on the expression of IL-22 and the transcription factor aryl hydrocarbon receptor (AHR). IL-22 can stimulate production of antimicrobial peptides by keratinocytes and Th22 cells can also recognise lipid antigens presented by Langerhans cells in the skin, suggesting these cells may be responsible for skin health and homeostasis (Zielinski et al., 2011). Th9 cells, which are characterized by IL-9 production, are specialized in the elimination of helminths and driven by the master regulator Pu.1 (Sallusto et al., 2009). Natural regulatory T cells (T_{reg}) are defined by expression of cell surface markers CD4 and CD25, as well as the intracellular transcription factor Foxp3. T_{reg} cells actively suppress activation of the

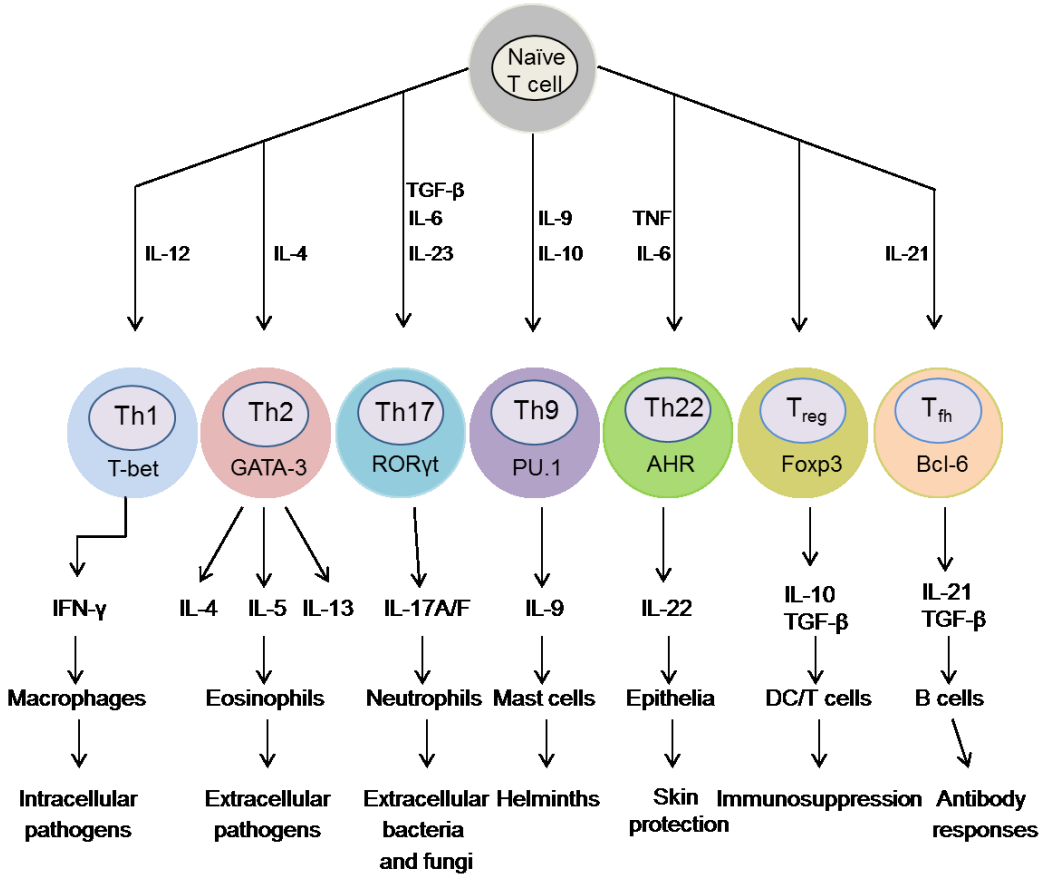


Figure 1.4. T helper cell subsets in adaptive immunity. Activated naïve $CD4^+$ T cells differentiate into different T helper (Th) cell subsets driven by cell-specific transcription factors and particular cytokines. The effector T helper subsets produce signature cytokines. The cytokines then target other immune cells to regulate immune responses against invading pathogens (modified from Zielinski et al., 2011).

immune system and prevent pathological self-reactivity, such as autoimmune disease (Schmitt et al., 2013). Some pathogens may have evolved to manipulate T_{reg} cells to immunosuppress the host to potentiate their own survival. IL-10 produced by T_{reg} cells favours the persistence of intracellular bacteria and the establishment of a pathogen carrier state, while deletion or blockade of IL-10 leads to clearance of parasites, such as *Leishmania major* in mice (Belkaid et al., 2001). Follicular Th cells (T_{fh}) are found within B cell follicles of lymphoid tissue and help memory B cell responses to antigen. They produce IL-21 and express the specific transcription factor Bcl-6 (Fazilleau et al., 2009).

1.2.3 The Th1/Th2 paradigm

The Th1/Th2 paradigm was first coined three decades ago by Mosmann and Coffman (Mosmann et al., 1986; Mosmann and Coffman, 1989) and has been central to our understanding of adaptive immune responses. Early innate immune response result in a cytokine environment, which can instruct naïve $CD4^+$ T cells to differentiate into at least two distinct subtypes, Th1 and Th2, to coordinate the immune response in clearance of the insulting pathogens. Th1 cells, through the secretion of IFN-γ, regulate immune responses against intracellular pathogens, such as viruses or certain bacteria. Th2 cells produce the signature cytokines IL-4, -5 and -13. Th2 responses are associated with protection against extracellular infections, such as with certain parasites (Murphy and Reiner, 2002). Th1 and Th2 cell differentiation is a complicated process (for details see Figure 1.5). The cytokines IL-12 and IL-4, through activation of signal transducer and activator of transcription 4 (STAT4) and STAT6 respectively, determine the outcome, respectively driving Th1 and Th2 cell differentiation. Costimulatory molecules and TCR on T cells can also

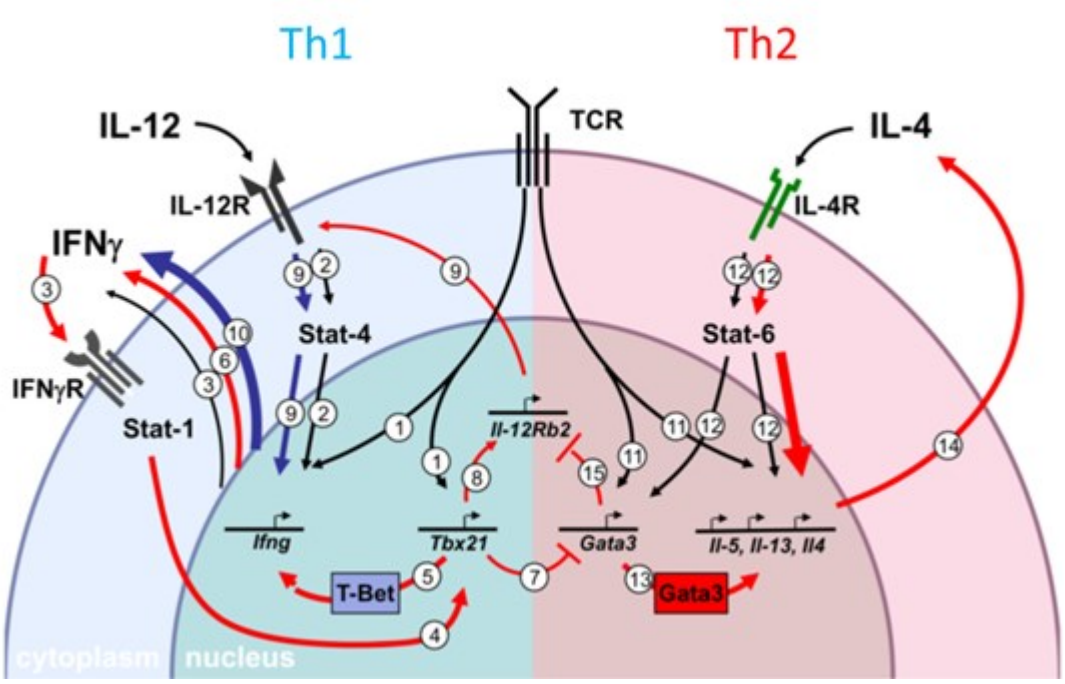


Figure 1.5. IL-12 and IL-4 drive Th1/Th2 cell differentiation.

Th1 induction by IL-12: initial TCR activation induces low level expression of IFN-γ and T-box21 (Tbx21 or T-bet) genes (1). IL-12 signalling results in activation of STAT4 to promote IFN-γ expression (2). IFN-γ receptor signalling activates STAT1 (3), which strongly promotes T-bet expression (4). T-bet then enhances transcription of IFN-γ (5) leading to increased production of this cytokine (6). In addition, T-bet prevents Th2 differentiation by inhibiting GATA-3 (7). T-bet also promotes expression of the IL-12 receptor β2 chain (8), resulting in greater IL-12 responsiveness (9) and yet further elevated production of IFN-γ (10).

Th2 induction by IL-4: initial TCR signalling induces low level expression of the IL-4 and GATA-3 genes (11). IL-4 receptor signalling strongly promotes expression of these two genes (12). GATA-3 then enhances transcription of IL-4, IL-5 and IL-13, (13). Increased IL-4 production further enhances Th2 cell differentiation (14). GATA-3 also prevents Th1 cell differentiation by inhibiting expression of the IL-12 receptor β2 chain (14). Primary events are indicated with black arrows, secondary events with red arrows and tertiary events with blue arrows (Amsen et al., 2009).

transduce the signals from APCs to positively modulate differentiation and proliferation of these cells (Amsen et al., 2009). Certain crucial transcriptional factors play key roles in the regulation of Th1/Th2 differentiation. The T-box transcriptional factor, T-bet, selectively responds to IL-12 stimulation and controls IFN-γ production as well as Th1 cell differentiation (Szabo et al., 2000). By contrast, the zinc-finger transcription factor, GATA-3, enhances the production of the Th2 cytokines IL-4, -5 and -13 (Hosoya et al., 2010). Accumulated IFN-γ and IL-4 regulate the differentiation of Th1 and Th2 in positive and negative feedback loops. Through IFN-γ signalling, IFN-γ not only reinforces Th1 cell differentiation, but also inhibition of GATA-3 activity to suppresses Th2 lineage development. By contrast, IL-4 positively regulates Th2 cell differentiation but suppresses Th1 cell development (Murphy and Reiner, 2002; Amsen et al., 2009).

1.3 Overview of the chicken immune system

Similarly to that of the human, the immune system in the chicken has evolutionarily developed an innate and adaptive response to cope with a wide diversity of pathogens. Although birds and mammals evolved from a common ancestor more than 300 million years ago, many aspects of mammalian immune responses also apply to the chicken immune system. Nevertheless, the chicken immune system has unique features, including certain lymphoid organs and a different immune cell repertoire.

The chicken also has a well-organised structure of lymphoid tissues (Figure 1.6), including both central and peripheral lymphoid tissues. The central lymphoid tissues include the bone marrow, thymus and bursa of Fabricius. Haemopoietic stem

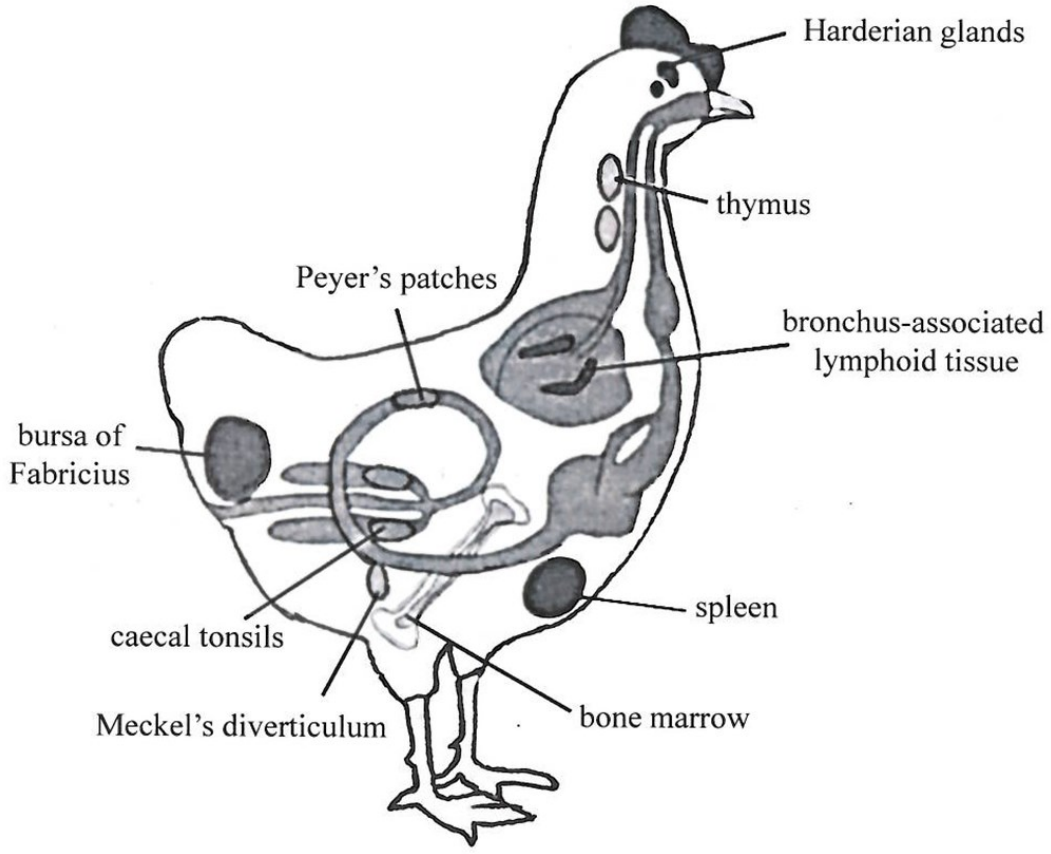


Figure 1.6. The chicken immune system. The central immune organs include the bone marrow for generation of immune cell progenitors, the thymus for T cell development and the bursa of Fabricius for B cell development. The peripheral immune organs include the Harderian glands, spleen, bronchus-associated lymphoid tissues and gut-associated lymphoid tissues (including Meckel's diverticulum, caecal tonsils, Peyer's patches).

cells in the bone marrow are the origins of peripheral lymphoid or myeloid cells. Immunologically mature chicken T cells develop in the thymus. Differently to mammalian B cell maturation in bone marrow, chicken B cells develop in the bursa, which is a unique lymphoid organ found only in avian species (Miller et al., 2002; Scott at al., 2004; Ratcliffe, 2006). In peripheral lymphoid tissues, the spleen is a key lymphoid organ as in mammals, but the chicken lacks encapsulated lymph nodes like those of mammals and instead has diffuse lymphoid tissues, mucosa-associated lymphoid tissues, which are widely present in the digestive, respiratory and reproductive systems. Some of these tissues have well-organised structures with T-dependent zones and B-dependent zones (also called germinal centres (GC)), such as the caecal tonsils, Meckel's diverticulum and Peyer's patches in gut-associated lymphoid tissues (GALT) and the Harderian gland behind the eye. Less-organised lymphoid tissues, such as nodules, aggregates of lymphocytes and a heterogeneous population of intra-epithelial leukocytes (IEL), are also present in mucosal areas and involved in immune responses (Befus et al., 1980; Jeurissen et al., 1989; Withanage et al., 1997; Casteleyn et al., 2010).

The first generation of macrophages is derived from the yolk sac, and probably acts more in organ development (Cuadros et al., 1992). After hatching, chicken macrophages originate from bone marrow stem cells. Bone marrow cells develop into a pro-monocyte and then into a monocyte under the influence of colony stimulating factor(s). Monocytes enter the blood stream where they constitute a major phagocytic cellular component. Blood monocytes migrate to a variety of the body's tissues and develop into macrophages (Qureshi, 2003).

Although identification of natural chicken DCs has been attempted, progress is limited. Chicken DCs differentiated from the bone marrow have been characterised. They possess similar characteristics as mammalian DCs in expression of functional molecules, such as PRRs, costimulatory molecules, MHC class II, cytokines, chemokines and chemokine receptors (Wu et al., 2010). As immune cells, they are competent in phagocytosis and endocytosis and once mature become potent activator of naïve T cells (Wu et al., 2010; 2011). Bone marrow-derived DCs also show anti-viral responses with increased cytokine production (Vervelde et al., 2013; de Geus et al., 2013).

Chicken heterophils, the counterparts of mammalian neutrophils, are polymorphonuclear granulocytes (Kogut et al., 1994; Stabler et al., 1994). Heterophils are important phagocytes of invading microbial pathogens. Activation of hererophils by pathogens induces pro-inflammatory cytokines and chemokines, such as IL-1β, IL-6 and CXCLi2 (Kogut et al., 2005). Chicken heterophils express a variety of TLRs to recognise pathogens, including both TLR2 variants, TLR4, TLR5, TLR7 and TLR21 (Kogut et al., 2005). Differently to mammalian neutrophils, chicken heterophils lack myeloperoxidase, resulting in less bactericidal activity by oxidative burst (Kogut et al., 2005). Furthermore, the chicken possesses nucleated thrombocytes, important phagocytes in the chicken but not present in mammals. Although the primary function of thrombocytes is in haemostasis, they are capable of phagocytosing bacteria and producing an oxidative burst response (Wigley et al., 1999). Thrombocytes may also play a role in innate immunity through the expression of a range of TLRs, resulting in increased pro-inflammatory cytokine production

following stimulation with agonists such as LPS and CpG oligonucleotides (St. Paul et al., 2012).

The chicken appears to lack functional eosinophils (Maxwell, 1987). Eosinophils in mammals are key components of allergic and anti-helminthic reactions, mediated by Th2 responses. There are many components of the mammalian Th2 response, and in particular those that control the response and function of eosinophils, are absent in the chicken (Kaiser, 2012). Th2 responses in mammals involve IL-4, -13 and -5, all of which are upregulated following infection with an extracellular pathogen. Amongst other functions, they drive antibody isotype switching towards IgA and IgE, the latter being crucial in eosinophil function as it binds the Fc-εR on eosinophils, triggering their degranulation. IgE production is driven by IL-5, after IL-4 induces isotype switching to IgE. In the chicken, IL-13 and IL-4 (the former up to 100-fold more than the latter) are both induced following infection with extracellular pathogens (Degen et al., 2005; Powell et al., 2009), but IL-5 expression is switched off (Powell et al., 2009). IgE is not produced in birds - chickens only have IgM, IgY (the functional equivalent of mammalian IgG), which does not have sub-types, and IgA. The eotaxins (chemokines) and the eotaxin receptor, which control migration of eosinophils in mammals, are absent in the chicken genome (Kaiser, 2012).

Chicken NK cells are of a different lymphoid lineage from T and B cells, and develop in a bursa- and thymus-independent manner. Chicken NK cells are defined as $CD3^{+}$ $CD8\alpha\alpha^{+}$ TCR^{-} MHC II^{-} Ig^{-} lymphocytes. They have large intracellular granules, with roles in spontaneous cytotoxicity (Roger et al., 2008). The number of

chicken NK cells in the blood, spleen and caecal tonsils is extremely low, ranging from 0.5 to 1% (Gobel et al., 2001); by contrast, in many mammals, 10% of the blood lymphocytes are NK cells (Cooper et al., 2001).

1.3.1 The chicken innate immune response

Similarly to mammals, the chicken innate immune system has its own receptors (PRRs) on effector cells (e.g. macrophages, DCs, heterophils and NK cells). The cytokines and chemokines resulting from an innate response drive inflammatory responses and influence subsequent adaptive responses through antigen-presenting cells (APC) presenting pathogen antigen to the adaptive immune response.

The best characterized family of chicken PRRs are the TLRs. There are ten chicken TLRs and six of these, TLR1, 2, 3, 4, 5 and 7, are clearly orthologous to mammalian TLRs (Brownlie and Allan, 2011; St. Paul et al., 2013). However, duplication of genes has led to the chicken having two TLR1s (TLR1a and b) and two TLR2s (TLR2a and b). Dimerisation between either of the two chicken TLR1 and TLR2 variants forms cell surface receptors to recognise at lease the same range of cell-surface PAMPs on pathogens as recognized by mammalian TLR2, which dimerises with either TLR1, 6 or 10 (Higuchi et al., 2008; Keestra et al., 2007). Similarly to mammals, chicken TLR3 (Iqbal et al., 2005) and TLR7 (Philbin et al., 2005) are intracellular receptors, and recognise pathogen RNA. However, TLR8 is a pseudogene, being disrupted by the insertion of a large CR1 repeat at some point during evolutionary history (Philbin et al., 2005). Murine TLR9 recognizes microbial DNA through unmethylated CpG motifs (Hemmi et al., 2000). Although chicken cells respond readily to CpG motifs (Vleugels et al., 2002; Xie et al., 2003; He et al.,

2006), TLR9 is absent from the chicken genome (Roach et al., 2005; Brownlie et al., 2009). There are two chicken-specific TLRs, TLR15 and TLR21 (Roach et al., 2005; Higgs et al., 2006). Chicken TLR21 has recently been shown to recognize CpG motifs (Brownlie et al., 2009; Keestra et al., 2010; St Paul et al., 2011). The mRNA expression levels of TLR15 were significantly increased in response to live, heat-killed or formalin-killed *Salmonella* Enteritidis, *Escherichia coli* or *Enterococcus gallinarum* (Nerren et al., 2010). In a separate study, its mRNA expression levels were significantly upregulated after induction with B- and C-type CpG oligonucleotides (ODN), tripalmitoylated lipopeptide (PAM3CSK4), *E. coli*- and Enteritidis-derived LPS in the chicken macrophage cell line, HD11 (Ciraci and Lamont, 2011). Together, this suggests that its ligand is a hitherto undescribed, heat-stable, non-secreted cell surface component of both $Gram^+$ and $Gram^-$ bacteria (Nerren et al., 2010), as would be predicted from the pattern of leucine-rich repeats in its extracellular domain (Kaiser, 2010; Kaiser et al., 2009). Recent evidence also indicated that TLR15 can be a sensor for secreted virulence-associated fungal and bacterial proteases and activation of TLR15 involves proteolytic cleavage of the receptor ectodomain and stimulation of NF-κB-dependent gene transcription (de Zoete et al., 2011). A yeast-derived agonist could also trigger TLR15 activation and resulted in TLR15-dependent activation of NF-κB (Boyd et al., 2012). *Mycoplasma synoviae*, specifically the NH_2-terminal diacylated lipopeptide (MDLP) representing the NH_2-terminal portion of its mature haemagglutinin protein, can also significantly upregulate the expression levels of TLR15 and activated TLR15 eventually triggers an increased expression levels of NF-κB and nitric oxide production (Oven et al., 2013).

The chicken appears to have a smaller repertoire of endocytic PRRs. For example, the chicken genome only contains NOD1, and NOD2 is absent. Chickens also lack RIG-I-like receptors (NLRs) (Kaiser, 2010). Recently, two cell surface C-type lectin receptors have been identified in chicken. DEC-205 was characterised and is expressed on chicken DCs and macrophages (Staines et al., 2013) and a partial cDNA of DC-SIGN (Acc. No. BU125096) has been identified in an EST library (Boardman et al., 2002; Schultz and Magor, 2013).

1.3.2 The chicken adaptive immune response

Chicken T and B lymphocytes, like their mammalian equivalents, play key roles in adaptive immune responses, through cell-mediated and humoral responses, to efficiently clear invading pathogens.

B cells use surface Igs as antigen receptors and differentiate into plasma cells to secrete antibodies. Differently to those of mammals, the chicken Ig light chain is encoded by a single copy of functional V_L and J_L genes. Therefore, diversity is very limited and the effects of VJ rearrangement are minimal (Reynaud et al., 1985). The chicken Ig heavy chain locus also possesses only single functional V_H and J_H genes, meaning little diversity can be generated through V(D)J rearrangement (Ratcliffe et al., 1994). However, clusters of pseudogenes, upstream of the heavy and light chain loci, are involved in somatic gene conservation to substitute V_L and V_H. The donor V pseudogenes (80-100 pseudo V_H and 26 pseudo V_L) effectively generate an enormous diversity of Ig variable regions (Ratcliffe et al., 1994). Activated chicken effector B cells produce three classes of antibodies, IgM, IgY (also called IgG), and IgA. IgM is the only isotype present on the surface of chicken B cell and is expressed

during B cell development in the bursa. Similarly to mammals, chicken IgA is predominantly a secretory Ig isotype. IgY is equivalent to mammalian IgG, but without subclasses. There are no equivalents of mammalian IgD and IgE documented in any avian species (Ratcliffe et al., 2006).

Chicken T progenitors differentiate into TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ T cell lineages in the thymus. TCR$\gamma\delta^+$ T cells can be distinguished by the antibody TCR1 (Sowder et al., 1988). In peripheral blood, TCR$\gamma\delta^+$ T cells do not express both of the co-receptors CD4 and CD8, whereas, when they migrate to the gut and spleen, the majority of TCR$\gamma\delta^+$ T cells express CD8 (Sowder et al., 1988; Davidson and Boyd, 1992), and a very low number of TCR$\gamma\delta^+$ T cells also express CD4 (Erf et al., 1998). Although the $\gamma\delta$ T cell frequency increases with age, even up to 50% of the circulating T cells in adult birds, their immunological functions are still poorly understood (Chen et al., 1996). Chicken TCR$\alpha\beta^+$ T cells have two subsets, expressing either $\alpha V\beta 1$ or $\alpha V\beta 2$, which can be distinguished by the antibodies TCR2 and TCR3 respectively (Davidson and Boyd, 1992). Mature TCR$\alpha\beta^+$ T cells express either CD4 or CD8. CD8 is composed of two chains, CD8α and CD8β, which can form two isoforms, CD8$\alpha\alpha$ and CD8$\alpha\beta$. As in mammals, most peripheral chicken $CD8^+$ T cells bear CD8$\alpha\beta$ heterodimers, whereas CD8$\alpha\alpha$ homodimers are expressed by the majority of chicken intestinal $CD8^+$ T cells (Imhof et al., 2000). Functionally, $CD8^+$ T cells serve as cytotoxic T cells, whereas $CD4^+$ T cells act as T helper cells. Through their secreted cytokines, $CD4^+$ T helper cells modulate the activity of cytotoxic $CD8^+$ T cells, NK cells, macrophages and other effector immune cells, including B cells in the humoral response (Zhu and Paul, 2008). Progress has been

made in determining if the chicken has similar range of the newly identified $CD4^+$ T helper subsets as seen in mammals. For example, the signature cytokines and cell surface markers for chicken Th17 (IL-17A, IL-17F, IL-21, IL-22 and IL-23R), Th9 (IL-9) and T_{reg} (IL-10, TGF-β4 and CD25) cells have been identified and characterised (Kaiser et al., 2005; Kaiser, 2010; Kaiser and Staheli, 2013). Certain cytokines are crucial in driving mammalian T helper lineage development. For example, TGF-β either with IL-6 and IL-23 or IL-4 can drive naïve $CD4^+$ T cells to differentiate into Th17 and Th9 respectively; TGF-β and IL-10 are critical for differentiation of inducible T_{reg} (also known as Th3 or Tr1); IL-21 is important for differentiation of the T_{fh} lineage (Cohn, 2008; Zhu and Paul, 2008). All of these cytokines are also present in the chicken. A precise spectrum of chicken $CD4^+$ T helper subsets will soon be unveiled with characterisation of the transcription factors, such as T-bet (Th1), GATA-3 (Th2) and RORγt (Th17), although Foxp3 (T_{reg}) remains to be identified in the chicken genome (Kaiser, 2010).

1.3.3 The chicken Th1/Th2 paradigm

The Th1/Th2 paradigm, originally defined according to different profiles of secreted cytokines in response to infection with intracellular or extracellular pathogens (Mosmann et al., 1986; Mosmann and Coffman, 1989), has been extended beyond mammals to the chicken, 20 years since the first chicken T helper cytokine, IFN-γ, was cloned (Digby et al., 1995). So far, chicken Th1/Th2 signature cytokines have been identified and characterised, such as Th1 cytokines, including IL-2 (Sundick et al., 1997; Kaiser et al., 1999; Rothwell et al., 2001), IFN-γ, IL-12 (Balu et al., 2003; Degen et al., 2004; Balu et al., 2011) and IL-18 (Schneider et al., 2000), and Th2 cytokines, including IL-4, IL-13 and IL-5 (Avery et al., 2004). Th1/Th2

cells critically determine the outcome of any given infection and direct the ongoing immune response through the secretion of cytokines, which act as growth and differentiation factors for themselves and other cell types (Szabo et al., 2003). The cytokines predominating adaptive responses in the chicken have been intensively investigated in infectious diseases, such as bacterial (Kogut et al., 2005; Beeckman et al., 2010), viral (Degen et al., 2005; Eldaghayes et al., 2006) and parasitic (Rothwell et al., 2004; Powell et al., 2009; Andersen et al., 2013) infections. Th1 cytokines predominate during infections with intracellular pathogens, and Th2 cytokines predominate during infections with extracellular pathogens, although, as in the pig, IL-13 seems to play a more important role than IL-4 in the chicken during Th2 responses (Degan et al., 2005; Powell et al., 2009; Raymond and Wilkie, 2004).

In mammals, T helper cell immune dysfunction leads to tissue injury and autoimmune disease. Although autoimmune diseases are not a major problem in domestic poultry, experimental observations in chicken disease models have shown Th1/Th2 dysregulation-related autoimmune diseases similar to those in mammals. Several laboratory-established chicken lines as animal models for autoimmune disease have manifested all of the clinical and biological symptoms of human disease. The Smyth line chicken (SL) is used to study the etiopathology of the autoimmune disease pigmentation disorder vitiligo. T cell infiltration of the feather pulp is involved in disease onset, with 15-times higher infiltration than those observed in feathers from non-vitiligous controls and the CD4:CD8 ratio decreases to levels below 0.4 (the ratio in non-vitiligous chickens is 1:1) (Wick et al., 2006). The Obese strain (OS) chicken, as a model of human Hashimoto's thyroiditis, has shown a role for IL-15 in driving the onset of spontaneous autoimmune thyroiditis (SAT)

and the Th1 cytokine, IFN-γ, plays a central role in inflammatory cell-mediated immune activities (Kaiser et al., 2002). Scleroderma, also known as systemic sclerosis (SSc), manifests in the UCD-200/206 chicken line, with a major role for T cells in the pathogenesis of the disease (Wick et al., 2006). Other autoimmune diseases caused by T cell dysregulation have been reported in the chicken, including experimental viral arthritis (Pertile et al., 1996), experimental allergic encephalomyelitis (Blaszczyk et al., 1978) and ovarian autoimmune disease (Barua and Yoshimura, 2001). These chicken autoimmune disease models also demonstrate that chicken Th1/Th2 cells are crucial in modulating self-immune tolerance.

1.4 The Tim gene family

As described before, distinguishing Th1 and Th2 cells is still largely based on their cytokine profiles and the expression of specific transcription factors. Classification of Th1/Th2 subsets based on cell surface phenotype has been difficult. Certain chemokine and costimulatory receptors are associated with Th1 and Th2 cells; however, expression differences are quantitative rather than qualitative (Zielinski et al., 2011; Sallusto et al., 2012). The lack of T helper subset-specific cell surface markers has limited their study.

A novel family of molecules, the T cell immunoglobulin and mucin domain (Tim) family, which encodes cell surface anchor proteins, are now known to be involved in the regulation of immune responses by modulating effector Th1 and Th2 cell functions. The Tim gene family has three members (Tim1, 3 and 4) in human (Kuchroo et al., 2003) and eight (Tim1-8) in mouse (Kuchroo et al., 2003; McIntire et al., 2001). Mouse Tim1-4 are known to be expressed but Tim5-8 are so far only

predicted genes (Kuchroo et al., 2003). Tim1 is predominantly expressed by activated Th2 cells with an important role to enhance T cell expansion and cytokine production (Umetsu et al., 2005). Tim3, a negative regulator of the Th1 response, is preferentially expressed by Th1 cells (Monney et al., 2002). Tim4, in contrast, is primarily found on APCs, and costimulates T cell proliferation by interacting with its natural ligand, Tim1 (Kuchroo et al., 2008; Mizui et al., 2008).

Tim1 is highly associated with asthma susceptibility in a simplified trait study for investigating asthma susceptible genes using mice as a model. The mouse Tim gene family was found by positional cloning, lying on chromosome 11B1.1. In human, the three Tim genes are encoded on chromosome 5q33.2, a region that has been linked with asthma, allergy and autoimmune disease (McIntire et al., 2004). There are no intervening genes in the human chromosome region, and there is conserved synteny between the human and mouse loci between the genes encoding IL-2-inducible T cell kinase (ITK) and a regulator of the transcription factor SP-1 (Crsp9). Murine Tim1, Tim3 and Tim4 are orthologues of human Tim1, Tim3 and Tim4 respectively, and therefore they have received the most attention to date (Meyers et al., 2005b).

1.4.1 The features of Tim family molecules

The Tim genes encode type I cell surface glycoproteins with common structural features, including an NH_2-terminal Ig-variable (IgV)-like domain, a mucin domain with O-linked glycosylation, a transmembrane domain and a cytoplasmic tail containing a tyrosine kinase phosphorylation motif, the latter absent in Tim4 (Figure 1.7A) (McIntire et al., 2001; Shakhov et al., 2004). The IgV domains of Tim proteins

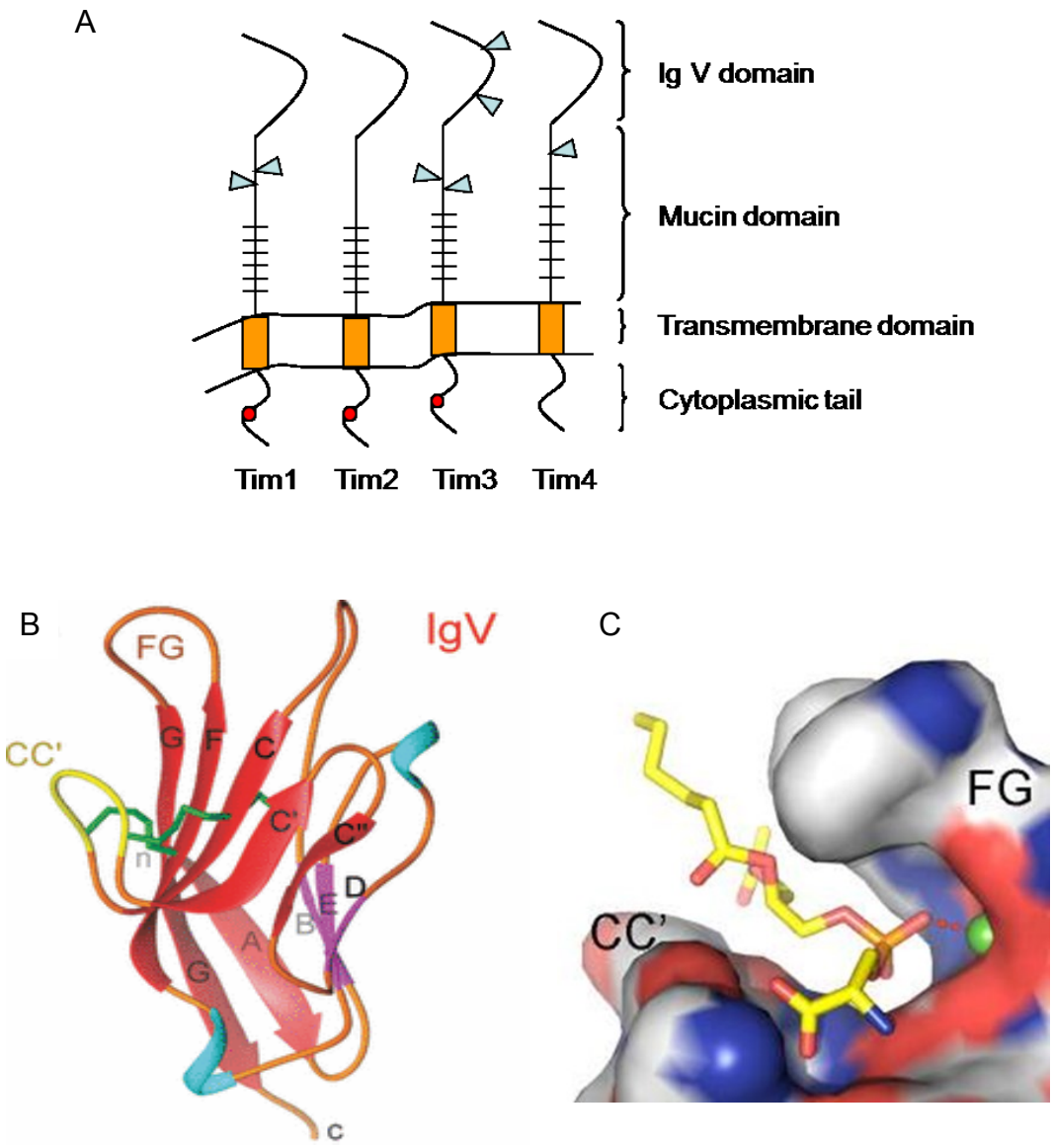


Figure 1.7. The features of Tim family proteins. (A) The mouse Tim family proteins, short lines are O-linked glycosylation sites, blue triangles are N-linked glycosylation sites. Red circles are tyrosine-phosphorylation sites (modified from Kuchroo et al., 2003). (B) Ribbon diagram of the mouse Tim IgV domain. β-strands of the GFC β-sheet are red and those in the BED face are pink, Cys residues and disulfide bonds are shown as green cylinders (Rofriguez-Manzanet et al., 2009). (C) Surface representation of the top region of the domain with bound PS and the sodium ion with which it interacts (green sphere). PS is shown in ribbon form (Santiago et al., 2007).

play a critical role in binding to their ligands, whereas the mucin domains have no detectable ligand but are thought to enhance the affinity of interaction of Tim molecules with their ligands (McIntire et al., 2001; Meyers et al., 2005a). Tim molecules are highly glycosylated proteins. Except for differential levels of N-linked glycosylation, the mucin domains of Tim molecules have abundant serine and threonine residues which serve as sites for O-linked glycosylation (Kuchroo et al., 2003).

The crystal structures of the Ig domains of four murine Tim family members have been determined (Cao et al., 2007; Santiago et al., 2007a; 2007b). The NH_2-terminal Tim IgV domains are composed of two anti-parallel β-sheets with particularly short β-strands, B, E and D in one sheet (BED β-sheet) and A, G, F, C, C' and C'' β-strands in the other sheet (GFC β-sheet). All Tim IgV domains contain six conserved Cys residues, and the first and last of these six Cys residues bridge the β-sheets, as the IgV domains in other Ig superfamily (IgSF) molecules. The four additional Cys residues form two additional disulphide bonds that fix the long CC'' loop upwards onto the GFC β-sheet (Figure 1.7B) (Freeman et al., 2010).

Co-crystalization of mouse Tim4 IgV with phosphatidylserine (PS) (Figure 1.7C) revealed it to be a PS receptor. The PS binding cavity is between the CC` loop and the neighbouring FG loop and there are four conserved amino acids (WFND) in this cavity with a critical binding role (Santiago et al., 2007a). PS is an indicator of apoptotic cells and is involved in the phagocytosis of apoptotic cells by macrophages. PS is normally expressed on the inner surface of a live cell's lipid bilayer membrane. During apoptosis, PS is exposed on the outer surface of the lipid bilayer membrane (Zwaal et al., 2005). The subsequent interaction of PS with Tim4,

expressed on the surface of DCs and macrophages, bridges clearance of the dead cell by the APCs (Santiago et al., 2007a).

Tim4-mediated phagocytosis was further proven by two other groups. Miyanishi et al. (2007) generated a library of hamster monoclonal antibodies against mouse peritoneal macrophages. One mAb, Kat5-18, strongly inhibited PS-dependent engulfment of apoptotic cells *in vitro*. The antigen recognized by this antibody was identified by expression cloning as Tim4. Kobayashi et al. (2007) also demonstrated that Tim4 binds PS and mediates uptake of apoptotic cells. Expression of Tim4 in fibroblasts (NIH-3T3 cells) resulted in efficient phagocytosis of apoptotic cells which could be blocked by an anti-Tim4 mAb (21H12). Mutations in the binding cavity of Tim4 eliminated PS binding and phagocytosis (Kobayashi et al., 2007). Anti-Tim4 mAbs (Kat5-18) that blocked PS binding and phagocytosis mapped to epitopes in this binding cavity (Miyanishi et al., 2007).

Tim1 shares similar properties to Tim4 (Kobayashi et al., 2007); it has a PS binding site in the IgV domain and Tim1-transfected 3T3 cells had increased uptake of apoptotic cells. Similar phagocytotic activity is also possessed by Tim3, although its PS-binding motif is slightly different to those of Tim1 and Tim4 (Nakayama et al., 2009). Tim2 seems to be the only member of the murine Tim family that does not mediate binding of PS (Santiago et al., 2007a; 2007b; Kobayashi et al., 2007). Tim1 and Tim3 are expressed on T cells, and it remains to be seen what the effects are of PS binding to these molecules on T cells, which are not phagocytes.

The cytoplasmic tails of Tim molecules, except for Tim4, possess a consensus tyrosine kinase phosphorylation site through which downstream signalling

pathways are initiated. There is a costimulatory role for Tim1 in T cell activation and differentiation under the stimulation of anti-Tim1 antibodies (Umetsu et al., 2005) or the interaction between Tim1 and Tim4 (the Tim1 receptor) (Meyers et al., 2005a). de Souza et al. (2005) reported that Tim1 could provide costimulatory signals for increased transcription of IL-4 through activation of the IL-4 promoter, but had no effect on IFN-γ production, and could also augment levels of nuclear factor of activated T cells and activator protein-1 (NFAT/AP-1), a critical transcription factor for Th2 development and IL-4 production, suggesting that Tim1 enhances Th2 cytokine production, consistent with the preferential expression of Tim1 on Th2 cells. Tim1 signalling was dependent on Y276 in the Tim1 cytoplasmic tail. The p85 subunit of phosphoinositide-3 kinase (PI3K) is recruited directly to Y276 by the p85 SH2 domain, after lymphocyte-specific protein tyrosine kinase (Lck)-dependent phosphorylation of the Tim1 cytoplasmic tail, and subsequently activates the downstream effector thymoma viral proto-oncogene (Akt) (de Souza et al., 2008). Tim1 therefore exerts an effect on NFAT/AP-1, at least in part, through the PI3K/Akt pathway.

Human Tim1 colocalizes on the T cell surface with CD3, and is recruited to the T cell receptor (TCR)-signalling complex (Binne et al., 2007). Stimulation with agonistic Tim1 mAbs led to rapid tyrosine phosphorylation of Tim1, phosphorylation of zeta-chain-associated protein kinase 70 (Zap-70) and IL-2-inducible T cell kinase (ITK), and recruitment of ITK and PI3K (Binne et al., 2007). ITK is crucial for commitment of T helper progenitors towards Th2 cells, with increasing expression levels during Th2 proliferation, and the ITK promoter contains binding sites for the

canonical regulator of Th2 differentiation, GATA-3 (Schwartzberg et al., 2005). Tim1 might therefore drive Th2 cell differentiation partially through activity of ITK.

The interaction of Tim1 with Tim4 in the presence of anti-CD3 and anti-CD28 mAbs leads to phoshorylation of Tim1, phosphorylation of thymoma viral proto-oncogene 1 (Akt1) and mitogen-activated protein kinase1/2 (Erk1/2), and induction of B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2), suggesting that Tim4 treatment can promote T cell survival (Rodriguez-Manzanet et al., 2008).

1.4.2 Tim1

Tim1 was originally identified in the African green monkey as a receptor for hepatitis A virus (HAV), named HAVCR1 and associated with virus invasion (Kaplan et al., 1996). Later, Tim1 was also described as kidney injury molecule 1 (Kim1), associated with kidney injury. Tim1 can act as a marker for acute kidney injury, because it is upregulated on renal epithelial cells and shed into the urine after kidney injury (Ichimura et al., 1998). Tim1 on renal epithelial cells plays an important role in tissue homeostasis by facilitating clearance of apoptotic and necrotic cells of injured tubules (Han et al., 2002; Ichimura et al., 2008). Tim1 is associated with kidney cancer, as Tim1 is upregulated in urine and tissue samples from patients with renal cell carcinoma (Vila et al., 2004).

In the immune system, Tim1 is expressed on activated but not naïve CD4 T cells (Umetsu et al., 2005). Following differentiation, Tim1 is mainly expressed on Th2 cells and regulates Th2-biased immune responses, while Th1 and Th17 cells express little or no Tim1 (Meyers et al., 2005a; Nakae et al., 2007b; Umetsu et al., 2005). NKT cells also express Tim1 on their cell surface, where it plays a similar

role as on CD4 T cells. Tim1 engagement on invariant NKT cells in the presence of TCR stimulation increased GATA-3 expression but reduced T-bet expression, resulting in enhancement of IL-4 production and inhibition of IFN-γ production (Kim et al., 2010). Murine Tim1 can be detected on the surface of peritoneal mast cells and bone marrow-derived cultured mast cells (Nakae et al., 2007a). In addition, *in vitro* activated murine B cells express low levels of Tim1 (Sizing et al., 2007).

Human Tim1 also acts as a cellular HAV receptor (Feigelstock et al., 1998) and is associated with susceptibility to asthma. An association study of HAV with asthma showed, before 1970, that the seroprevalence of antibodies against HAV approached 100% in western countries, and infection with HAV may have protected many individuals against atopy (Matricardi et al., 2002). However, significant improvements in public health have caused anti-HAV seroprevalence to fall to 25-30%, while the prevalence of atopic disease has doubled. Interestingly, HAV infection is merely a marker of poor hygiene, because it is transmitted through the faecal-oral route. As HAV is not a respiratory pathogen, how can it regulate development of atopy disease (Umetsu and Dekruyff, 2004)? A hygiene hypothesis was proposed to explain the association of Tim1 with atopy. HAV might interact with Tim1 on T cells, reduce Th2 cell differentiation, and thereby diminish the likelihood of developing asthma and other atopic diseases (McIntire et al., 2003; 2004; Bach, 2002). Polymorphisms in murine Tim1 genes have shown association with asthma and atopic disease. In DBA/2 and C57/BL6 mouse strains, there are major single nucleotide polymorphisms (SNPs) and deletion variants in the Tim1 mucin domain, explaining the tendency of these strains to develop reduced Th2 and air-way hyperreactivity (AHR) responses compared to the AHR susceptible BALB/c

stain (McIntire et al., 2001). Genetic studies of human Tim1 also revealed that the insertion of six amino acids (157insMTTTVP) and a depletion of one amino acid (195delT) were associated with protection from atopy, but this protection was only observed in individuals with HAV seropositivity (McIntire et al., 2003), which further supports the hygiene hypothesis. A similar pattern of association between Tim1 and atopy has also been confirmed in studies of large populations from different human genetic backgrounds (Chae et al., 2003; Gao et al., 2005; Page et al., 2006; Wu et al., 2009). However, one study in a Japanese population, where the incidence of HAV infection is close to zero, showed an absence of association of Tim1 with asthma (Freeman et al., 2010).

Several ligands for Tim1 have been identified. Except for the above described hepatitis virus A and PS, Tim1 also binds to itself (Santiago et al., 2007), IgAλ (Tami et al., 2007), and leukocyte mono-immunoglobulin (Ig)-like receptor 5 (LMIR5)/CD300b (Yamanishi et al., 2010). Crystal structure analysis revealed the homophilic murine Tim1-Tim1 interaction is through the BED faces of the two Tim1 IgV domains (Santiago et al., 2007). IgAλ binds Tim1 on a different site to HAV and their association has a synergistic effect in virus-receptor interaction (Tami et al., 2007). The Ig-like domain of LMIR5 binds to Tim1 in the vicinity of the PS-binding site within the IgV domain of Tim1 and this interaction plays a physiological role in immune regulation by myeloid cells (Yamanishi et al., 2010).

1.4.3 Tim3

Tim3 was originally characterised as having a specific expression pattern on Th1 but not on Th2 cells through a screen for Th1-specific cell surface markers

(Monney et al., 2002). Tim3 is expressed on cytotoxic $CD8^+$ T cells (Wang et al., 2007) and at low levels on Th17 cells (Nakae et al., 2007a; Hastings et al., 2009), monocytes, DCs (Anderson et al., 2007), macrophages and mast cells (Frisancho-Kiss et al., 2006; Nakae et al., 2007b).

Galectin-9 was identified as a receptor for Tim3, recognising a carbohydrate structure on the IgV domain of Tim3 (Zhu et al., 2005). Tim3, through its ligand galectin-9, negatively regulates Th1 immunity. Galectin-9-induced intracellular calcium flux, aggregation and death of Th1 cells were Tim3-dependent *in vitro*, and administration of galectin-9 *in vivo* resulted in selective loss of IFN-γ-producing cells and suppression of Th1 autoimmunity (Zhu et al., 2005). These suppression effects on Th1 cells had decreased severity of diseases caused by Th1-mediated immunity. In a murine experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis (MS), *in vivo* administration of galectin-9 fusion protein selectively reduced the number of IFN-γ-producing cells and resulted in ameliorated disease severity (Zhu et al., 2005). Galectin-9 showed effects on amelioration of collagen-induced murine arthritis through inhibition of the generation of Th17 cells and induction of T_{reg} cells (Seki et al., 2008). In transplantation models, *in vivo* administration of galectin-9 prolonged survival of fully mismatched cardiac allografts through suppression of Th1 and Th17 immune responses (He et al., 2009), and improved survival of fully allogeneic skin grafts though activation of the Tim3-galectin-9 pathway (Wang et al., 2008). Consistent with its role as an inhibitory molecule, blockage of Tim3 resulted in an exacerbation of autoimmune disease. *In vivo* administration of specific mAb to murine Tim3 during induction of EAE deteriorated disease progression and resulted in higher numbers of activated

macrophages (Monney et al., 2002). In a non-obese diabetic mice model, Tim3 pathway blockade, through treatment with either a Tim3-Ig fusion protein or an anti-Tim3 mAb, accelerated the onset of diabetes (Sanchez-Fueyo et al., 2003). Moreover, *in vivo* administration of mTim3-Ig fusion protein to mice, previously immunised with antigen, caused hyperproliferation of Th1 cells and increased production of IFN-γ, explained as the interaction of Tim3 with its ligand inhibiting effector Th1 cells during a normal immune response, whereas disruption of this pathway resulted in impaired peripheral tolerance (Sabatos et al., 2003).

As mentioned earlier, innate immune cells, such as DCs, macrophages and mast cells, also express Tim3. Tim3 was rapidly expressed on mast cells and macrophages in mice following viral infection, whereas treatment with anti-Tim3 mAb resulted in decreased activities of mast cells and macrophages with downregulation of CD80, suggesting Tim3 could be involved in the early stages of immune responses against pathogen invasion (Frisancho-Kiss et al., 2006). However, cross-linking Tim3 on the surface of mast cells with anti-Tim3 antibody increased IL-4, IL-6 and IL-13 production, resulting in an enhancement of Th2 immunity (Nakae et al., 2007b). When compared with wild type DCs, DCs from Tim3-deficient mice less efficiently produced the inflammatory cytokine tumor necrosis factor (TNF)-α, after LPS and galectin-9 stimulation, suggesting an agonistic effect was present between Tim3 pathway and TLR signalling (Anderson et al., 2007). During induction of EAE, cross-linking Tim3 with anti-Tim3 antibody worsened disease progression, explained as triggering Tim3 on macrophages leading to promotion of inflammatory Th1 responses (Monney et al., 2002). So far, the data suggest that Tim3 plays distinct roles in innate and adaptive immunity. Tim3 on cells

of the innate immune response synergizes with TLR signals to activate these cells, which in turn drive Th1 and Th2 responses. However, Tim3 can also terminate Th1 cell responses in adaptive immunity through its receptor galectin-9.

Tim3 is highly expressed on exhausted $CD8^+$ T cells in chronic viral infections to suppress the immune function of these cells. $CD8^+$ T cell exhaustion describes a state of T cell dysfunction after chronic viral infection, with loss of capability to proliferate and effector function, such as cytotoxicity and cytokine secretion in response to antigen stimulation (Moskophidis et al., 1993; Zajac et al., 1998). Tim3 is upregulated on $CD8^+$ T cells from HIV patients. These Tim3-expressing cells failed to produce cytokines or proliferate in response to antigen. However, blocking the Tim3 signalling pathway restored proliferation and enhanced cytokine production in HIV-1-specific T cells (Jones et al., 2008). Similar phenomena with Tim3 expression on exhausted $CD8^+$ T cells were also observed in patients with chronic hepatitis B (HBV) (Ju et al., 2010) and C (HCV) (Golden-Mason et al., 2009) virus infection. Tim3-expressing cells were the most abundant fraction among virus-specific $CD8^+$ T cells. With treatment with antibody against Tim3, exhausted $CD8^+$ T cells can be partially recovered to proliferate and produce IFN-γ (Golden-Mason et al., 2009; Ju et al., 2010).

Allelic variants in mouse Tim3 are clustered in the Ig domain and are thought to affect interactions between PS and Tim3, resulting in variable PS binding affinity and phagocytic capacities. An allele of Tim3 in BALB/c mice bound to PS with higher affinity and mediated more efficient phagocytosis of apoptotic cells than an allele of Tim3 in HBA mice (DeKruyff et al., 2010). Two alternative splice variants of murine Tim3 were also identified, which encoded a membrane-anchored protein

and a soluble protein with only the IgV domain. The membrane-bound Tim3 had normal roles as described previously. However, the function of soluble Tim3 is not clear; it may also be involved in Tim3-mediated immune responses but in a competitive way to bind Tim3 ligand (Sabatos et al., 2003). Genetic studies showed SNPs in human Tim3 were associated with idiopathic thrombocytopaenic purpura disease (Du et al., 2009) and type 1 diabetes (Bruck et al., 2008).

1.4.4 Tim4

Unlike other members of the Tim family in mouse and human, Tim4 is not expressed on T cells but is instead expressed on APCs, particularly on DCs and macrophages (Meyers et al., 2005a). Tim4 plays a role in regulation of T cell responses. Administration of Tim4-Ig fusion protein increased basal T cell proliferation and cytokine production (Meyers et al., 2005a). However, *in vivo*, Tim4-induced T cell activation seems to be in a dose-dependent manner. For mice treated with high-dose of Tim4-Ig fusion protein, *in vitro* analysis of their splenocytes indicated high levels of IL-2 and IFN-γ production, whereas there were high levels of IL-4 and IL-10 production from low-dose Tim4-Ig treated mice (Meyers et al., 2005a). Tim4 also plays bimodal roles in the regulation of naïve and activated T cell responses. Tim4-Ig fusion protein bound to naïve $CD4^+$ T cells through an unknown ligand and inhibited activation and proliferation of these cells (Mizui et al., 2008; Cao et al., 2011). However, for pre-activated $CD4^+$ T cells, Tim4 plays a role in costimulation of these cells in the presence of anti-CD3 and -CD28 antibodies (Meyers et al., 2005a).

Murine Tim4 was found to be a ligand of murine Tim1 using a stably transfected CHO cell model, where CHO transfectants overexpressing Tim1, Tim3 or Tim4 on the cell surface were stained with various Tim fusion proteins (Tim1-Ig, Tim2-Ig and Tim4-Ig) (Meyers et al., 2005a). Tim4-Ig bound to Tim1 transfectants but not to Tim3 or Tim4 transfectants. Conversely, Tim1-Ig bound to Tim4 transfectants but not to Tim1 or Tim3 transfectants. This interaction could be blocked by a specific anti-Tim1 mAb (Meyers et al., 2005a). Therefore, the Tim4-induced proliferation of pre-activated $CD4^+$ T cells was thought to be through surface expressed Tim1 on activated cells, because Tim1, as a receptor of Tim4, is upregulated on activated T cells (Meyers et al., 2005a; Mizui et al., 2008). A Tim4-Ig fusion protein also stained higher proportions of *in vitro*-polarised Th2 cells than Th1 cells (Meyers et al., 2005a), which is consistent with the observation that Tim1 is specifically expressed on Th2 cells. Tim4-induced T cell activation resulted in the phosphorylation of Tim1, along with increased phosphorylation of key signalling components for the activation of T cells, Akt1 and Erk1/2 (Rodriguez-Manzanet et al., 2008).

However, binding with highly purified proteins failed to detect a direct interaction between Tim1 and Tim4 (Sizing et al., 2007). Contrary findings were also reported by Wilker et al. (2007). Murine Tim1, Tim3 and Tim4 fusion proteins non-specifically bound to the surface of cells overexpressing murine Tim1, Tim3 and Tim4, and authors interpreted these results as homotypic and heterotypic Tim interactions. Miyanishi et al. (2007) suggested exosomes may bridge Tim4/Tim1 interaction, because Tim1 and Tim4 can bind to separate sites on the surface of an

exosome. Exosomes expose PS at their outer leaflet and Tim proteins might serve as receptors for exosomes via PS.

In addition, Tim4 lacks a tyrosine in its cytoplasmic tail, suggesting Tim4 lacks the ability to transduce signals. Studies have confirmed that Tim4 does not mediate direct downstream signalling, as a version of Tim4 without its cytoplasmic tail still promoted the uptake of apoptotic cells via PS recognition (Park et al., 2009). Cross-linking Tim4 expressed on RAW264.7 cells, a murine macrophage cell line, with Tim1-Ig fusion protein can regulate cytokine production and B7 family member expression (Hein and Woods, 2007). All these findings suggest that Tim4-Tim1 interactions might transduce activation signals one-way through Tim1. If two-way transduction occurs, Tim4 would need other transmembrane protein(s) to transmit a cytoplasmic signal.

1.4.5 Tim2

Tim2 is present in mouse, but not present in human. Similarly to Tim1, Tim2 expression was upregulated on activated T cells, and exclusively maintained on the surface of differentiated Th2 cells (Kumanogoh et al., 2002; Chakravarti et al., 2005). Germinal centre B cells in the spleen have high surface expression levels of Tim2 (Chen et al., 2005). Tim2 has two different ligands, H-ferritin and Sema4A. H-ferritin is secreted by macrophages in a soluble form. Interaction of Tim2 with H-ferritin increased internalisation of H-ferritin and had a physiological role in the regulation of iron mineralisation and sequestration (Chen et al., 2005). Sema4A is a class IV semaphore, expressed in DCs and B cells, and plays a role in the regulation of T cell responses (Kumanogoh et al., 2002). Tim2 seems to be a specific regulator

of Th2 immune responses. Administration of Tim2-Ig fusion protein resulted in the preferential induction of Th2 cell responses, inhibition of Th1 responses, and administration of Tim2-Ig fusion protein during the induction phase reduced the severity of EAE (Chakravarti et al., 2005). Sema4A-dificient mice resulted in dysregulated Th cell proliferation and impaired Th1 responses, suggesting that Tim2 served to downregualte establishment of Th2 responses (Kumanogoh et al., 2005). Other evidence also showed that Tim2 plays a role in suppressing Th2 responses. $CD4^+$ T cells from Tim2-deficient mice showed higher levels of proliferation in response to antigen stimulation than those of wild-type cells and markedly higher levels of IL-5, IL-10, and IL-13, suggesting the inhibitory activity of Tim2 was turned off, resulting in an Th2 over-response (Rennert et al., 2006). Tim2 overexpressed in T cell lines also showed a significantly impaired induction of NFAT/AP-1 transcription reporters, suggesting that Tim2 signalling induces inhibitory activities in T cell responses (Knickelbein et al., 2006).

1.4.6 Tim molecules in other animals

So far there have been no reports of Tim family molecules in species beyond human and mouse, resulting in a suggestion by Ohtani et al. (2012) that the Tim family are mammalian-specific molecules. Analysis of Tim molecules in 24 different primates, including Prosimians, New World monkeys, Old World monkeys and Hominoids (including human), indicated that Tim1 became a pseudogene in multiple lineages of the New World monkey, caused by the insertion of deleterious sequences or base substitutions leading to a termination codon. In other monkeys, Tim1 has undergone positive selection in the IgV domain (with more variations) during the course of primate evolution. However, in Old World monkeys, more polymorphisms

can be seen in the mucin domain, suggesting they might be under balanced selection. By contrast, no significant positive selection occurred in the Tim3 and Tim4 genes in all primates (Ohtani et al., 2012).

1.5 Hypothesis and aim of this project

Mammalian Tim family of molecules are specifically expressed on Th1, Th2 and APCs and play immunological and physiological roles on these cells. Blasting searches of chicken genome found only chicken Tim1 and Tim4. Like mammals, chicken Th1/Th2 cells by producing distinct cytokines determine the outcomes of intracellular and extracellular pathogens. A hypothesis is that chicken Tim molecules may evolutionarily conserved certain functions as those of their mammalian counterparts, such as specific expression patterns on immune cells and biological roles in phagocytosis of dead cells and costimulatory activity; on the other hand, mammalian Tims, especially Tim1, are highly associated with autoimmune diseases, while autoimmune diseases are rare events in chicken, implying diverse roles of Tim molecules may be present between chicken and mammal species.

Therefore, the aim of this project is to clone and characterise the Tim family genes in the chicken and to determine if they have similar biological activities, and specific expression patterns on the surface of chicken immune cells, as their mammalian equivalents.

Chapter 2. Materials and Methods.

2.1 General reagents

2.1.1 General chemicals

General chemicals were of analytical or molecular biology grade supplied by Sigma-Aldrich (Poole, UK) or Invitrogen (Paisley, UK).

2.1.2 Solutions

All solutions were made up in Milli-Q (reverse osmosis; ion-exchanged; activated charcoal filtration) water unless otherwise stated. All solutions for RNA-related work were made in RNase-free water or 0.1% diethyl pyrocarbonate (DEPC)-treated water.

2.1.3 Vectors

1. pGEM-T Easy	Used in TA cloning (Promega)
2. pKW06-Ig	A kind gift from John Young (Institute for Animal Health, Compton, UK) (Figure 2.1)
3. pKW06-his6	Constructed by replacing the Ig tag in pKW06-Ig with a his6 tag (Figure 2.2)
4. Signal pKW06-Ig	A kind gift from John Young (Institute for Animal Health, Compton, UK) (Figure 2.3)

2.1.4 Antibodies

Antibodies used in this study are shown in Table 2.1.

Antigen	Species	Clone/Subclass	Conjugation	Supplier
chCD4	Mouse	CT-4/IgG1	-----	SouthernBiotech
chCD4	Mouse	CT-4/IgG1	FITC	SouthernBiotech
chBu-1	Mouse	AV20/IgG1	-----	SouthernBiotech
chBu-1	Mouse	AV20/IgG1	FITC	SouthernBiotech
chCD28	Mouse	AV7/IgG1	-----	SouthernBiotech
chCD3	Mouse	CT-3/IgG1	-----	SouthernBiotech
Chicken monocyte/ macrophages	Mouse	KUL01/IgG1	-----	SouthernBiotech
Chicken monocyte/ macrophages	Mouse	KUL01/IgG1	FITC	SouthernBiotech
Rat PDI	Mouse	RL90/IgG2a	-----	Thermal Scientific
Rat GM130	Mouse	35GM130/IgG1	-----	BD Biosciences
Penta-his	Mouse	IgG1	-----	QIAGEN
huIgG	Rabbit	Polyclonal	FITC	SouthernBiotech
huIgG	Goat	Polyclonal	HRP	SouthernBiotech
huIgG	Goat	Polyclonal	Biotin	SouthernBiotech
huIgG	Goat	Polyclonal	-----	SouthernBiotech
mIgG	Goat	Polyclonal	Alexa fluor 647	SouthernBiotech
mIgG	Goat	Polyclonal	Alexa fluor 488	SouthernBiotech
mIgG	Goat	Polyclonal	HRP	SouthernBiotech
Biotin	Streptavidin	-----	Alexa fluor 568	Invitrogen

Table 2.1. Antibodies used in this study. Ch: chicken; hu: human; m: mouse.

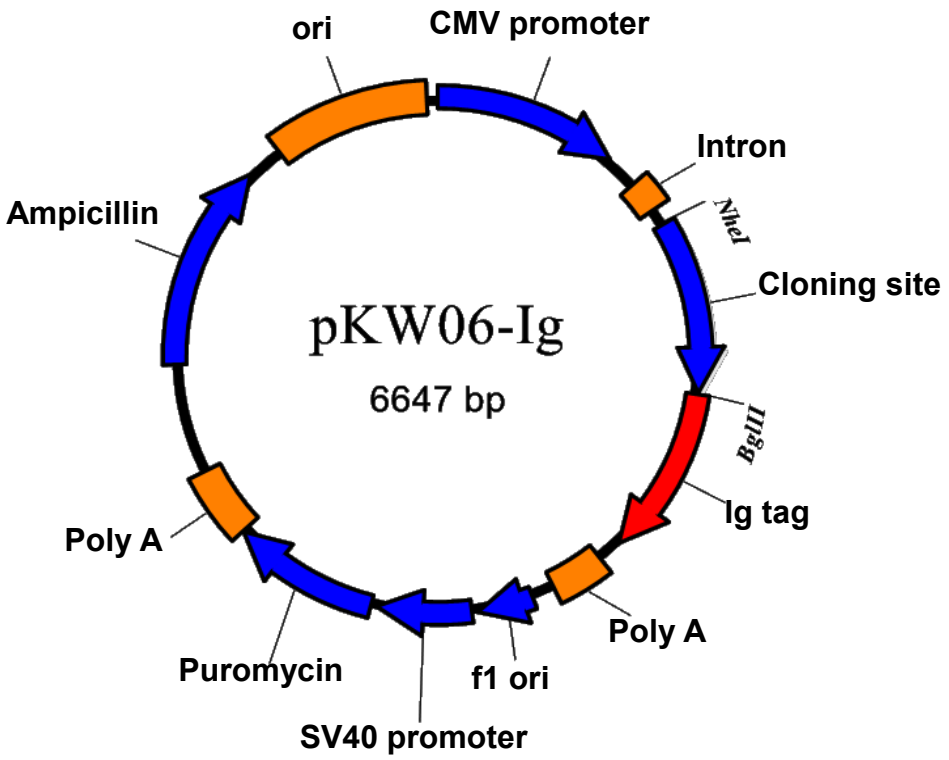


Figure 2.1. pKW06-Ig vector map. This is a mammalian expression vector with a COOH-terminal human IgG Fc tag. cDNAs to be expressed are cloned between *Nhe*I and *Bgl*II enzyme sites, with their own signal peptide for expression of the fusion protein.

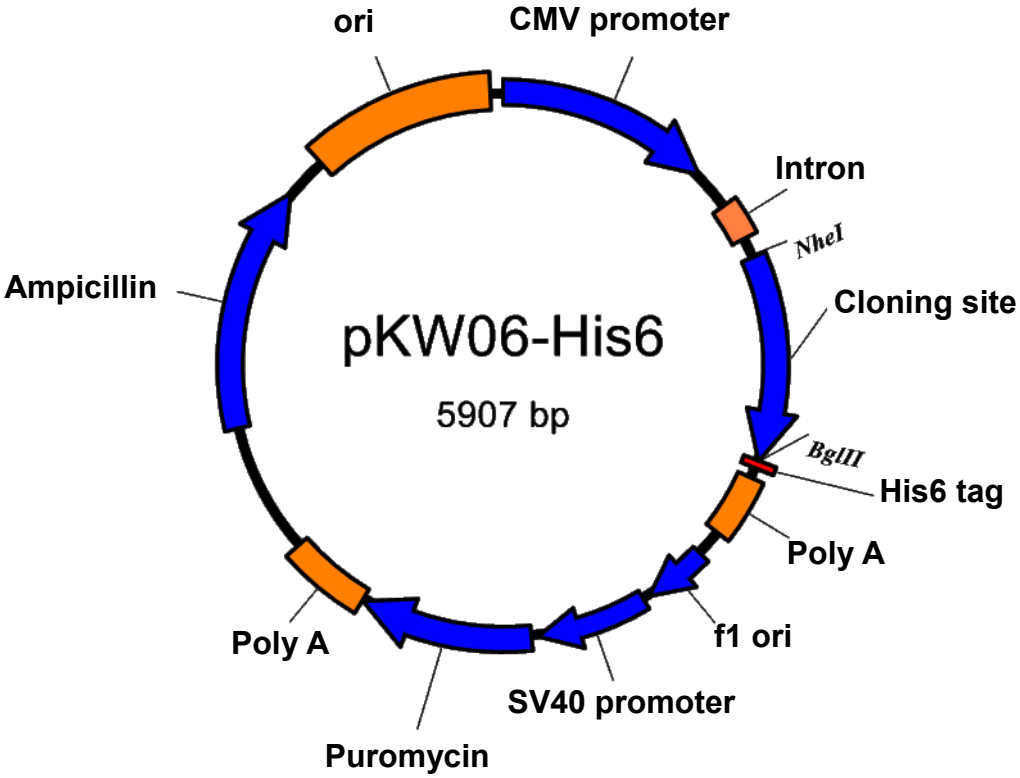


Figure 2.2. pKW06-His6 vector map. This is a mammalian expression vector with a COOH-terminal His6 tag. cDNAs to be expressed are cloned between *Nhe*I and *Bgl*II enzyme sites, with their own signal peptide for expression of the fusion protein.

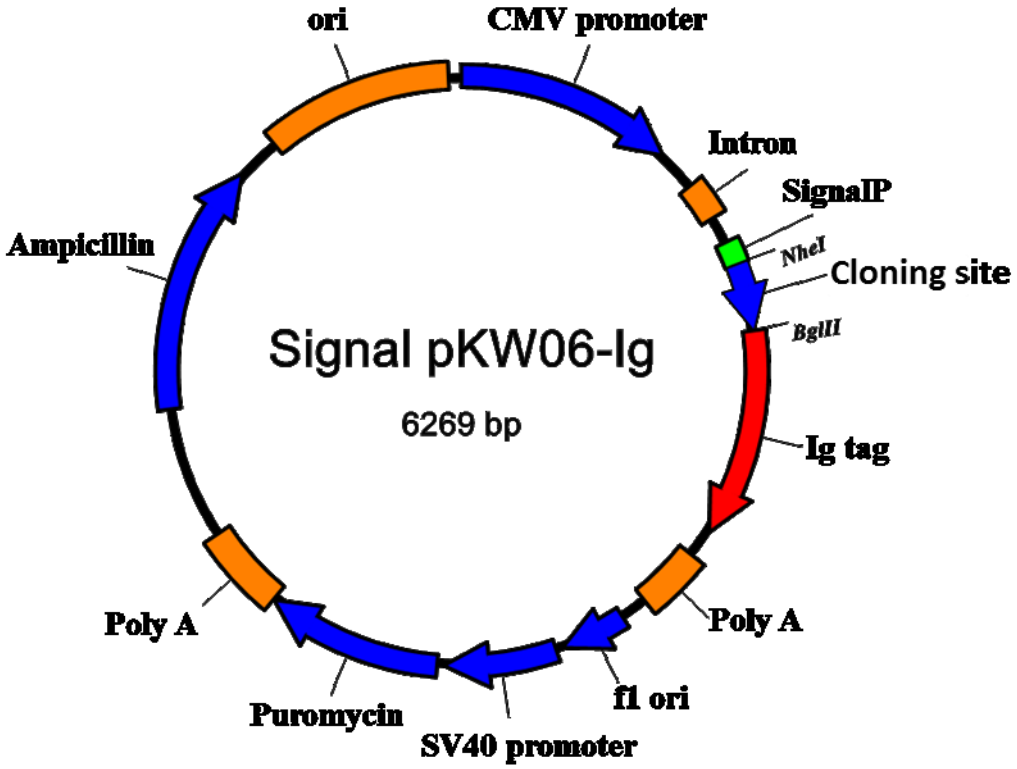


Figure 2.3. Signal pKW06-Ig vector map. This is a mammalian expression vector with a signal peptide from the mouse CD8α gene (green box) and a COOH-terminal human Ig Fc tag. cDNAs to be expressed are cloned between *Nhe*I and *Bgl*II enzyme sites for expression of the fusion protein.

2.2 Animals

All the animals used in the work reported in this thesis were handled and killed in accordance with the Animals (Scientific Procedures) Act 1986.

2.2.1 Chickens

The chickens used in this study were Brown Leghorn J line birds, bred in the poultry unit, The Roslin Institute, or specified-pathogen-free (SPF) inbred line 7_2 chickens, bred in the Poultry Production Unit at the Institute for Animal Health, Compton, UK.

2.2.2 Mice

Biozzi or BALB/c strain mice were isolated in cages with free access to food and water.

2.3 Cells

2.3.1 Reagents for cell culture

Cell culture media were purchased from Gibco Life Technologies (Paisley, UK) or Sigma-Aldrich (Poole, UK). 100× penicillin-streptomycin containing 5,000 Units of penicillin and 5 mg of streptomycin per ml, 100× L-glutamine (200 mM), 100× non-essential amino acids (NEAA), 100 mM sodium pyruvate, foetal bovine serum (FBS, EU-approved, South American origin), chicken serum (CS, New Zealand origin), 2.5% trypsin and 1× versene were all purchased from Gibco Life Technologies. 0.4% Trypan-blue solution was obtained from Sigma-Aldrich. FBS and CS were thawed and incubated at 56ºC for 30 min in a water-bath for heat-inactivation of serum.

2.3.2 General conditions for maintenance of mammalian cell lines

COS-7 and NIH-3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), CHO cells in Ham's F12 medium and SP2/0 in RPMI 1640 medium. All cell growth media were supplemented with 10% FCS, 2 mM L-glutamine, 1× NEAA and 1 mM sodium pyruvate unless otherwise stated. Mammalian cell lines were cultured at 37ºC in a humidified incubator with 5% CO_2.

To resurrect the cell lines from liquid nitrogen storage, the cells were thawed at 37ºC in a water-bath, washed once with cell growth medium and resuspended in 15 ml of this medium. The cell suspension was then transferred to a T75 tissue culture flask and returned to a CO_2 incubator. Three days later, when the cells were fully confluent, they were ready for passaging. Adherent cells, such as COS-7, 3T3 and CHO, were detached from the flask by trypsinisation. In brief, the cell layer was washed once with PBS, 5 ml of 0.25% trypsin/versene mix were then added to cells, incubating at 37ºC for 5 min. Tapping the flask to loosen the cells, 10 ml of cell growth medium were added to the flask to stop trysinization and the cell suspension was collected in a Universal. Pelleting cells by spinning at 1,000 *g* for 4 min, the cells were resuspended in the respective cell growth medium. To count cells, a small aliquot of the cell suspension was mixed with an equal volume of 0.4% trypan blue solution, and counted with a haemocytometer. Cells were seeded at 7.5×10^5 cells in 15 ml cell growth medium in a T75 flask and cultured at 37ºC, 5% CO_2. Semi-adherent cells, such as SP2/0, were detached from the flask by shaking the flask vigorously and the cell suspension was collected in a Universal for pelleting the cells. Cells were seeded at 2×10^6 cells in 15 ml cell growth medium in a T75 flask and cultured at 37ºC, 5% CO_2.

2.3.3 General conditions for isolation and culture of chicken primary cells

All chicken tissues for cell isolation were removed aseptically post-mortem, placed in Universals in cold sterile PBS and delivered to the laboratory on ice or ice packs in a polystyrene box. Chicken primary cells were cultured in complete RPMI 1640 medium supplemented with 8% FCS, 2% CS, 50 Units/ml penicillin, 50 μg/ml streptomycin, 1× L-glutamine, 1× NEAA and 1× sodium pyruvate unless otherwise stated, and incubated at 41ºC in a humidified incubator with 5% CO_2.

2.3.4 Cell lines

2.3.4.1 COS-7 cells

The COS-7 cell line is an African Green Monkey kidney cell line. It is an adherent cell line derived from the CV-1 cell line by transformation of an origin-defective mutant of SV40 virus that can produce large T antigen but not replicate itself. When a plasmid with an SV40 promoter is introduced into COS-7 cells, the vector can be replicated substantially by the large T antigen, enabling transient expression of a recombinant gene (Gluzman, 1981).

2.3.4.2 CHO-K1 cells

CHO-K1 cells were derived from the ovary of a Chinese hamster. The CHO cell line is an adherent cell line with a requirement for proline in its growth medium due to the lack of the gene for proline synthesis. This cell line possesses a small number of chromosomes (2n = 22) and is normally used as a model system in the quantitative study of the genetic action of various agents on cells cultivated *in vitro*

(Tjio, 1958). CHO cells are the most commonly used mammalian hosts to express recombinant protein, especially when long-term and high yields of proteins are required.

2.3.4.3 NIH-3T3 cells

3T3 cells come from a cell line established in 1962 by George Todaro and Howard Green. 3T3 cells were originally obtained from Swiss mouse embryonic tissue. This cell line has become the standard fibroblast cell line. The '3T3' refers to the fact that primary mouse embryonic fibroblast cells were transferred (the "T") every 3 days (the first "3"), and inoculated at the rigid density of 3×10^5 cells per 20 cm^2 dish (the second "3") continuously. Spontaneously immortalized cells with a stable growth rate were established after 20-30 generations in culture (Todaro, 1963).

2.3.4.4 SP2/0 cells

Sp2/0-Ag14 was isolated as a re-clone of Sp2/HL-Ag, itself derived in several steps from Sp2/HLGK, a hybrid between a BALB/c spleen cell contributing a γ2b (H) and κ (L) chains with anti-sheep red blood cell activity and the myeloma cell line X63-Ag8. Sp2/0-Ag is resistant to 20 μg/ml 8-azaguanine, dies in HAT-supplemented medium and does not synthesise Ig chains. It has about 73 chromosomes which is only eight more than the chromosome number of X63-Ag8, a cell line commonly used to generate hybridomas (Shulman, 1981).

2.3.5 Chicken primary cells

2.3.5.1 Isolation of chicken splenocytes

Spleens were sampled from J line or line 7_2 chickens aged 4-8 weeks. For splenocyte isolation, in brief, the spleen was placed in a petri dish with 10 ml cold PBS. A single cell suspension was prepared by repeatedly squeezing the spleen with forceps. The cell suspension was carefully aspirated by plastic pipette, passed through a 100 μm pore size cell strainer (BD Bioscience, Oxford, UK) and collected in a 50 ml Falcon tube. The cells were pelleted by centrifugation at 1,500 *g* for 10 min. To remove the red blood cells, the cell pellet was resuspended in 5 ml Histopaque 1083 (Sigma-Aldrich), and then 5 ml PBS carefully layered on top. The tube containing the cell suspension was centrifuged at 2,000 *g* for 25 min without braking. The white fluffy layer at the PBS and Histopaque interface can be visualized after centrifugation. This layer was carefully collected and transferred to a new Universal. The cells were washed by adding 15 ml PBS to the cells and pelleted by centrifugation at 1,500 *g* for 10 min. The cells were resuspended in complete RPMI 1640 medium, and then counted.

2.3.5.2 Preparation of B cells from the bursa of Fabricius

Bursae of Fabricius were sterilely removed from J line chickens aged 4 weeks old or less, because younger birds have larger bursae than older birds. The bursae was then placed in a petri dish with 10 ml cold PBS and cut into small pieces. These pieces were repeatedly squeezed with forceps to release single bursal cells. The cell suspension was collected and passed through a 100 μm pore size cell strainer to remove big tissue clumps. The cells were then pelleted by centrifugation at 1,500 *g*

for 10 min. The cell pellet was resuspended in complete RPMI 1640 medium and then counted.

2.3.5.3 Isolation of chicken bone marrow cells

Femur bones were taken from J line chickens aged 4-8 weeks. The ends of the femur bones were cut off and the bones placed in a petri dish. The bone marrow was flushed out with a 21G needle attached to a 20 ml syringe, which was filled 15 ml RPMI 1640. Repeatedly flushing of the bones released as many single cells as possible. The cell suspension was then collected, passed through a 100 μm cell strainer and collected in a Universal. The Universal was centrifuged at 1,500 *g* for 10 min at 4ºC to pellet the bone marrow cells. Removing the supernatant, the cell pellet was resuspended in 10 ml of Histopaque 1.077 (Sigma-Aldrich) and 10 ml PBS were carefully overlaid. After centrifugation at 2,000 *g* for 30 min without braking, a fluffy layer can be seen at the interfaces between the Histopaque layer and PBS. This cell layer was carefully aspirated with a plastic pipette, transferred to a 50 ml Falcon tube, topped up to 50 ml by PBS, mixed thoroughly and the cells pelleted by centrifugation at 1,500 *g* for 10 min. The cells were resuspended in complete RPMI 1640 medium and then counted.

2.3.5.4 Stimulation of chicken splenocytes

Splenocytes or bursal cells were prepared as described before (section 2.3.5.1 and 2.3.5.2) and cultured in complete RPMI 1640 medium. To stimulate the cells, 5 ml cell suspension at a cell density 2×10^6 cells/ml were added to a T25 flask with or without 3 μg/ml Concanavalin A (ConA), 5 μg/ml phytohaemagglutinin (PHA) or 1

μg/ml phorbol-12-myristate-13-acetate (PMA), and incubated at 41ºC, 5% CO_2. The duration of culture depends upon the experiment, as detailed in the Results Chapters.

2.3.5.5 Preparation of chicken bone marrow-derived macrophages

Recombinant chicken CSF-1 (rchCSF-1) fused with a COOH-terminal His6-V5 tag was transiently expressed from COS-7 cells (section 2.7.1) and a dot-blotting assay (section 2.6.3) was utilized to estimate the protein concentration. To obtain bone marrow-derived macrophages, the bone marrow cells, prepared as described in section 2.3.5.3, were suspended and adjusted to 2×10^6 cells/ml in complete RPMI 1640 medium. The cell suspension (15 ml) was added to a Sterilin square tissue culture dish (Thermal Scientific) with the addition of 75 μl COS-7 supernatant containing rchCSF-1 and incubated at 41ºC, 5% CO_2 for 6 days, changing the media for fresh rchCSF-1 medium every two days. The cells were harvested by repeatedly blowing the cell layer with a sterile plastic pipette. Transferring the cell suspension to a Universal, the cells were pelleted by centrifugation at 1,500 *g* for 10 min. The cell pellet was resuspended in an appropriate volume of complete RPMI 1640 medium and then counted.

2.3.5.6 Chicken bone marrow-derived dendritic cells (DCs)

Recombinant chicken IL-4 (rchIL-4) and GM-CSF (rchGM-CSF) were transiently expressed from COS-7 cells as described in section 2.7.1. The bone marrow cells, prepared as in section 2.3.5.3, were suspended and adjusted to 2×10^6 cells/ml in complete RPMI 1640 medium. Cell suspension (5 ml) supplemented with 25 μl rchIL-4 and 25 μl rchGM-CSF was added to each well of 6-well plates and incubated at 41ºC, 5% CO_2 for 6 days, changing the media for fresh rchIL-4 and

rchGM-CSF medium every two days. The cells were harvested as described in section 2.3.5.5

2.3.6 Bacterial strains

E.coli strain JM109: endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, Δ(lac-proAB), [F´ traD36, proAB, laqIqZΔM15], was used for routine propagation of plasmids.

2.4 RNA manipulation

Chicken tissue samples were preserved in *RNAlater* (Ambion, Huntingdon, UK) for 1 week at 4ºC to stabilize RNA as per the manufacturer's instructions, prior to isolation of total RNA, using an RNeasy Mini kit (QIAGEN, East Crawley, UK) according to the manufacturer's instructions. In brief, the tissues were cut and weighed. Each sample (25-30 mg) was transferred to a 2 ml safe-lock Eppendorf tube with a 5 mm stainless steel bead. Lysis buffer RLT (600 µl) was added to the sample, and the tissue was thoroughly disrupted and homogenized on a bead mill, Mixer Mill MM 300 (RETSCH, Hunslet, UK), for 4 min at 20 Hz. The lysate was pipetted onto a QIAshredder spin column and the column was centrifuged at 10,000 *g* for 2 min. For total RNA from cells, 600 µl RLT buffer were added to 5×10^6 pelleted cells, vortexing vigorously to lyse the cells. An equal volume of 70% ethanol was added to tissue or cell lysate and mixed thoroughly by pipetting. This mixture was then transferred to an RNeasy spin column and the column was centrifuged at 10,000 *g* for 1 min. After washing the column with 700 µl buffer RW1 once and 500 µl RPE twice, the column was then removed and placed into a new 1.5 ml collection tube. To elute the RNA, 50 µl RNase-free water were added to the spin

column membrane, incubated for 1 min at room temperature and centrifuged at 10,000 *g* for 1 min. RNA samples were stored at -20ºC until later use.

2.5 Molecular Biology Methods

2.5.1 First-strand cDNA synthesis

First-strand cDNA was synthesised using SuperScript^{TM} II (Invitrogen). Following the manufacturer's instructions, briefly, 5 µg of spleen total RNA from a 4-weeks-old line 7_2 chicken were mixed with 1 µl 500 µg/ml oligo $(dT)_{12-18}$, 1 µl 10 mM dNTP mix, bringing the total volume to 12 µl with DEPC-treated water. The mixture was heated to 65^{o}C for 5 min and immediately chilled on ice. The tube was briefly centrifuged to collect the contents on the tube wall. The follows were then added: 4 µl 5× first-strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 M DTT and 1 µl RNase inhibitor (40 Units/µl), gently mixed and incubated at 42^{o}C for 2 min. One µl SuperScript^{TM} II (200 Units) was added and gently mixed by pipetting. The tube was incubated at 42^{o}C for 50 min and the reaction was stopped by heating at 75^{o}C for 15 min.

2.5.2 Polymerase chain reaction (PCR)

The PCRs were performed on a Mastercycler Thermo cycler (Eppendorf, UK) using Invitrogen's recombinant *Taq* DNA polymerase purified from *E. coli* expressing a cloned *Thermus aquaticus* DNA polymerase gene. According to the manufacturer's instructions, the reaction mixture contained 2 µl 10× PCR buffer minus Mg^{2+}, 0.4 µl 10 mM dNTP mixture, 0.6 µl 50 mM $MgCl_2$, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 0.2 µl *Taq* polymerase (5 Units/µl), 1 µl cDNA

template and H_2O to 20 µl. Cycling conditions were: denaturation at 94°C for 3 min, then amplification with 25 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 2 min. The PCR was extended for 10 min at 72°C, and the sample stored at 25°C. This standard PCR condition was used to subclone target cDNA from a TA cloning vector into an expression vector, or for colony PCR to screen positive bacterial colonies. For particular cDNA amplification, the PCR conditions will be described in the relevant Results Chapters.

2.5.3 DNA gel electrophoresis

PCR products were resolved on a 1.0% agarose gel (made up with 0.5× TBE buffer, Ambion, UK) containing 1× GelRed (Cambridge Bioscience, Cambridge, UK). Electrophoresis was at a constant voltage of 150 V for 1 h in 0.5× TBE buffer. The resolved DNA was visualised on a UVP dual-intensity UV transilluminator.

2.5.4 DNA fragment extraction

A QIAquick gel extraction kit (QIAGEN) was used to extract DNA fragments from agarose gels according to the manufacturer's instructions. Briefly, the target DNA band was excised from an agarose gel and placed in a 1.5 ml Eppendorf tube, 3 gel volumes of buffer QG added and heated at 50ºC for 10 min until the gel slice was completely dissolved in buffer. One gel volume of 100% isopropanol was added to the gel solution and mixed. The mixture was transferred to a spin column and centrifuged at 10,000 *g* for 1 min. The column was washed once with 750 µl buffer PE. Finally, the DNA bound on the column was eluted by the addition of 20 µl Milli Q water.

2.5.5 TA cloning

DNA fragments extracted from agarose gels were cloned into a TA cloning vector, pGEM-T Easy (Promega, Southampton, UK). The ligation mixture contained 5 µl 2× rapid ligation buffer (60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG), 1 µl (3 Units) T4 ligase, 1µl vector and 3 µl DNA to be cloned, gently mixed and incubated at 16ºC overnight.

2.5.6 Transformation of ligation products into competent cells by heat-shock

Competent *E. coli* JM109 cells (Promega) were retrieved from a -80ºC freezer and thawed on ice. Ligation product (5 µl) was mixed with 50 µl competent cells in a sterile 1.5 ml Eppendorf tube and incubated on ice for 20 min. The cells were then heat-shocked at 42ºC for 45 s and returned to ice again for 5 min, followed by the addition of 1 ml SOC medium (Microbiology Services, IAH Compton) and incubation at 37ºC for 1 h in a shaking incubator at 200 rpm. LB agar plates supplemented with 100 µg/ml of ampicillin (Microbiology Services, IAH Compton) were spread with 40 µl 40 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma-Aldrich) and 100 µl 100 mM isopropyl-β-thiol-galactopyranoside (IPTG) (Sigma-Aldrich) for blue/white colony selection. The transformed competent cells were pelleted with centrifugation at 7,000 *g* for 3 min and resupended in 100 µl SOC, 50 µl of which were then spread on the X-gal and IPTG agar plates. The plates were incubated at 37ºC overnight.

2.5.7 Isolation of plasmid DNA

White colonies were picked, incoculated into 5 ml LB broth (Microbiology Services, IAH Compton) in Universals and cultured at 37ºC overnight in a shaking incubator at 200 rpm. A QIAGEN QIAprep spin miniprep kit was used to isolate plasmid DNA according to the manufacturer's instructions. Briefly, the bacterial cultures were centrifuged at 3,000 *g* for 30 min. The bacterial pellet was resuspended in 250 µl P1 buffer and transferred to a 1.5 ml Eppendorf tube. P2 buffer (250 µl) was added to lyse the bacterial suspension, mixed by inverting the tube 4-5 times, followed by the addition of 350 µl N3 buffer to stop the lysis reaction. The tubes were centrifuged at 10,000 *g* for 10 min. The supernatant was decanted to a spin column. The column was centrifuged at 10,000 *g* for 1 min. After washing the column once with 750 µl buffer PE, the plasmid DNA bound to column was eluted with 50 µl Milli-Q water.

2.5.8 DNA sequencing

In-house DNA sequencing was performed using a Dye Terminator Cycle Sequencing (DTCS) Quickstart kit (Beckman Coulter, High Wycombe, UK) for the sequencing reaction and read on a Beckman Coulter Capillary Sequencer CEQ8000. For each reaction, 200 ng of plasmid DNA were diluted to 15.5 µl with Milli-Q water, heated at 95ºC for 1 min and immediately cooled down on ice. The following reagents were then added to the PCR mixture: 1 µl 4 pmol sequencing primer, 2 µl Quickstart reagent and 1.5 µl reaction buffer. The thermal cycling was performed as follows: denaturation at 96ºC for 30 s, annealing at 50ºC for 30 s and extension at 60ºC for 4 min. A stop solution was made up with 2 volumes 100 mM EDTA

(Sigma-Aldrich), 2 volumes 3 M sodium acetate (Sigma-Aldrich) and 1 volume glycogen (provided in the kit). For clean-up of the reaction product, 5 µl of stop solution were firstly added to the reaction, the PCR product was then precipitated by the addition of 60 µl pure ethanol, mixed thoroughly and centrifuged at 10,000 *g* for 15 min at 4ºC. The pellet was washed with 200 µl 70% ethanol twice, air-dried and then resuspended in 40 µl sample loading solution (SLS).

2.5.9 Restriction enzyme digestion

Restriction enzyme sites were introduced to ends of cDNA fragments by PCR (section 5.2.1) via primer pairs designed to contain a *Nhe*I at the 5' end of the forward primer and a *Bgl*II enzyme site at the 5' end of the reverse primer. The PCR product was then ligated into a TA cloning vector (section 2.5.5), followed by transformation of competent *E. coli* JM109 cells and blue/white screening (section 2.5.6). Plasmids from positive clones were prepared at a small scale (section 2.5.7), and subjected to DNA sequencing (section 2.5.8) to confirm the DNA sequence of the insert in these plasmids.

A plasmid containing the expected cDNA sequence with enzyme sites was subjected to double restriction enzyme digestion. The digestions were carried out separately due to the fact that *Nhe*I and *Bgl*II utilise different buffers. Briefly, 1 µg plasmid DNA was digested with *Nhe*I enzyme by mixing 2 µl 10× NEBuffer 2 (100 mM Tris-HCl, 500 mM NaCl, 100 mM $MgCl_2$, 10 mM dithiothreitol), 1 µl *Nhe*I (10 Units) (NEB, Herts, UK), 0.2 µl 100 µg/ml BSA and Milli-Q water to 20 µl. After incubation at 37ºC for 2 h, the buffer and enzyme were removed using a QIAquick PCR purification kit (QIAGEN), according to the manufacturer's instructions. Buffer PB (100 µl) was added to the digestion mixture, mixed and then transferred to a spin

column. After centrifugation at 10,000 *g* for 1 min, the column was washed once with 750 µl buffer PE. The DNA bound to the column was eluted with 20 µl Milli-Q water. The *Bgl*II enzyme digestion was performed as follows: 15 µl eluted DNA, 3 µl 10× NEBuffer 3 (500 mM Tris-HCl, 1M NaCl, 100 mM $MgCl_2$, 10 mM dithiothreitol), 1 µl *Bgl*II (10 Units) (NEB), and 11 µl Milli-Q water. Mixing thoroughly, the mixture was incubated at 37ºC for 2 h.

To separate the target cDNA fragment from the digested mixture, the digested product was resolved on a 1% agarose gel as described in section 2.5.3 and extracted as described in section 2.5.4.

2.5.10 Ligation of gene fragment with sticky ends into expression vector

The above double-enzyme digestion was also applied to the relevant mammalian expression vectors, maps of which are shown in section 2.1.3. To construct the expression plasmids, cDNA fragments were ligated in these expression vectors by mixing the following components: 5 µl 2× rapid ligation buffer (Promega), 1 µl (3 Units) T4 ligase (Promega), 3 µl cDNA fragment and 1 µl digested vector, gently mixed and then incubated at 16ºC overnight.

2.5.11 Large-scale preparation of endotoxin-free plasmid DNA

Endotoxin-free plasmids were prepared using an Endo-free plasmid Maxi kit purchased from QIAGEN. According to the manufacturer's instructions, briefly, bacteria containing plasmids of interest were inoculated into 100 LB broth with the addition of 100 µg/ml of ampicillin and cultured overnight at 37ºC on a shaking incubator at 200 rpm. The cells were pelleted and resuspended in 10 ml resuspension buffer P1. To lyse the cells, 10 ml lysis buffer P2 were added to the cell suspension,

mixed and incubated for 5 min at room temperature. Finally, 10 ml buffer P3 were added to stop the reaction and release the plasmids from the bacterial genomic DNA and protein. A QIAfilter maxi cartridge barrel was used to remove the genomic DNA and protein from the lysate, the flowthrough was collected in a 50 ml Falcon tube, followed by the addition of 2.5 ml buffer ER and incubation on ice for 30 min to remove the endotoxin. Meantime, a QIAGEN-tip 500 column was equilibrated by passing through 10 ml buffer QBT. The ER-treated plasmid solution pass through the column by gravity and plasmids bind to the column. After washing the column twice with 30 ml wash buffer QC, the plasmid DNA was eluted with 15 ml buffer QN. To precipitate the plasmid DNA, 10.5 ml isopropanol were added to the elution solution. The DNA was then pelleted by centrifugation for 1 h at 4,000 *g*. After washing the DNA pellet twice with 2.5 ml 70% ethanol and drying, the plasmid DNA was resuspended in an appropriate volume of sterile Milli-Q water.

2.6 Protein detection and analysis methods

2.6.1 Capture enzyme-linked immunosorbent assay (ELISA)

The capture antibody, an unlabelled goat anti-human IgG polyclonal antibody, was diluted to 1 μg/ml in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate), added to 96-well microtitre plates, 50 μl/well, and incubated at 4ºC overnight. The plates were washed three times and blocked with 100 μl/well 1% casein/PBS (w/v) for 1 h at room temperature. The plates were washed again and recombinant protein to be measured was added to the plates, 50 μl/well, and incubated for 1 h. The plates were washed again as before. 0.5% casein/PBS solution was added to the plates at 50 μl/well and then the primary antibodies, mouse anti-

chicken antibodies, were titrated on the plates, along with a negative control antibody. After incubation for 1 h and washing the plates again, the HRP-conjugated goat anti-mouse IgG antibody, 1 µg/ml dilution in 0.5% casein/PBS, 50 µl/well, was added to the plates and incubated for 1 h. Meanwhile, O-phenylenediamine dihydrochloride (OPD) substrate solution was prepared by dissolving an OPD tablet (Invitrogen) in 10 ml of substrate buffer, composed of 5 ml Milli-Q water, 2.5 ml 0.2 M disodium hydrogen phosphate and 2.5 ml 0.1 M citric acid, prior to use, adding 10 µl of 30% hydrogen peroxide (H_2O_2). After the plates were washed again, 50 µl/well of substrate solution was added to the plates and incubated for 2 min. The reactions were stopped by the addition of 50 µl/well of 2N H_2SO_4. Optical density (OD) was measured on an ELISA microtitre plate reader at a wavelength of 490 nm.

2.6.2 Modified capture ELISA

A modified capture ELISA assay was designed to test the presence of recombinant IgG Fc protein expressed in tissue culture supernatants. The capture antibody, an unlabelled goat anti-human IgG polyclonal antibody, was coated onto 96-well micro-titre plates as described in section 2.6.1. After blocking the plates as described in section 2.6.1, 50 µl per well of 0.5% casein/PBS solution were added to the plates and then tissue culture supernatants containing chicken Ig fusion proteins were titrated on the plates; negative controls were cell culture medium only, positive controls were purified IgG Fc fusion protein diluted in cell culture medium to different concentrations, e.g. 1, 5 and 10 µg/ml. The plates were incubated for 1 h at room temperature, followed by three washes. A goat anti-human IgG antibody conjugated with HRP (1:1,000 dilution in 0.5% casein/PBS, 50 µl/well) was added to the plates, and incubated for 1 h. The colorimetric reaction of HRP was detected by

OPD substrate as before. This method was also used to estimate protein expression levels. Recombinant Ig protein in the supernatant should bind to the capture antibody at the same ratio as the positive controls, so that identical concentrations of protein in supernatant and positive control will produce the same optical density. Based on this, the fusion protein concentration in a supernatant can be roughly calculated by comparison with that in the positive control.

2.6.3 Dot- or slot-blotting assays to detect expression of His6 fusion proteins

A Hibri-Dot 96 device (Whatman) was used to isolate protein on nitrocellulose membrane. The device was assembled by laying a membrane support plate on a waste reservoir plate, followed by a sheet of nitrocellulose membrane to cover every hole on the support plate. Finally, a cover plate with 96 wells and a sample reservoir was carefully placed on the membrane. When all three plates were in the correct position, they were tightened by 8 screws.

Protein samples were added to wells on the cover plate of the Hibri-Dot 96. A vacuum connector in the waste reservoir plate was linked to a vacuum pump. When the vacuum pump was switched on, the protein solution was aspirated from the sample reservoir in the cover plate, through the membrane and then the holes in membrane support plate, finally collecting in the waste reservoir plate. The membrane was removed, washed in PBST buffer for 30 min and blocked in 1% casein/PBS solution for 1 h on a rocking shaker. The membrane was then transferred into a Penta-His antibody solution, 1:2,000 dilution in 1% casein/PBS, and incubated for 1 h at room temperature on the rocking shaker. After three washes with PBST, 10 min per wash on the rocking shaker, a goat anti-mouse IgG-HRP antibody, 1:1,000

dilution in 1% casein/PBS, was added to the membrane, incubating for 1 h on the rocking shaker. The membrane was washed three times again as before. The binding secondary Ab was detected using enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (GE Healthcare), as per the manufacturer's instructions. Equal volumes of luminol substrate and peroxide solution from the ECL kit were directly mixed before use, homogeneously distributed on the membrane, incubated for 5 min and dried with tissue paper. The membrane was developed by autoradiography. A digital image of the autoradiograph was taken by scanning the film with a scanner (EPSON Perfection V700).

2.6.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved in 4-12% discontinuous polyacrylamide gels using a Mini-PROTEAN Tetra system (Bio-Rad). To cast a polyacrylamide gel, a resolving gel monomer solution was prepared by combining the following reagents to make a 12% gel: 3.4 ml Milli-Q water, 4 ml 30% acrylamide/Bis, 2.5 ml 4× resolving gel buffer, 0.1 ml 10% SDS. Prior to casting the gel, 0.1 ml 10% APS and 10 µl TEMED were added and mixed gently to avoid bubbles. The solution was smoothly poured into the space between the two glass plates, which were assembled on a casting stand. The surface was immediately overlaid with water-saturated isobutanol. The gel was allowed to polymerise for 45 min. A 4% stacking monomer solution was prepared by mixing the following reagents: 6.1 ml Milli-Q water, 1.3 ml 30% acrylamide/Bis, 2.5 ml 4× resolving gel buffer, 0.1 ml 10% SDS. As before, prior to casting the gel, 0.1 ml 10% APS and 10 µl TEMED were added. After completely removing the overlay solution, the stacking gel solution was continuously poured until the surface reached the top of the short glass plate. The desired comb was then

inserted into the space between the glass plates. When the stacking gel had polymerised, the gel was mounted into the electrode assembly, lowered into the Mini tank, and the tank filled with 1× running buffer (3.0 g Tris base, 14.4 g glycine, 1.0 g SDS dissolved in 1 litre Milli-Q water).

2× SDS sample buffer was made as follow: 1) for non-reducing buffer, 1.25 ml 0.5 M Tris/HCl, pH6.8, 2.5 ml glycerol, 2.0 ml 10% SDS, 0.2 ml 0.5% bromophenol blue with H_2O to 9.5ml; 2) for reducing buffer, 50 µl β-mercaptoethanol were added to 950 µl of the non-reducing buffer. Protein samples were prepared by mixing equal volumes of protein solution and 2× SDS-PAGE sample buffer and then heated at 95°C for 5 min. Gel electrophoresis was carried out at a constant voltage of 150 V for 1 h. The gel was then stained with Coomassie blue or the protein was transferred to nitrocellulose membrane for western blot analysis.

2.6.5 Coomassie blue staining

To visualise separated proteins in an SDS-PAGE gel, Coomassie Brilliant Blue R-250 (National Diagnostics, Hessle, UK) was used to stain the gel. This is an anionic dye, which non-specifically binds to proteins. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by destaining buffer. The proteins are detected as blue bands on a clear background. Coomassie blue staining solution was made by dissolving 0.25 g Coomassie Brilliant Blue R-250 in 45 ml methanol, and then adding 45 ml H_2O and 10 ml acetic acid, stirring on a magnetic stirrer for 1 h to ensure the Coomassie blue dye was completely dissolved and finally filtering the solution through Whatman No 1 filter paper to remove particles of undissolved dye. The gel was immersed in Coomassie blue staining solution in a suitable container and incubated for 1 h on a

rocking shaker. The gel was then briefly rinsed with Milli-Q water and transferred to Coomassie destaining buffer (30 ml methanol, 10 ml acetic acid, 60 ml H_2O). The wet gel was then scanned with an EPSON scanner to generate a digital image.

2.6.6 Western blotting

After protein samples were resolved by electrophoresis on an SDS-PAGE gel, the slab gel was equilibrated in trans-blotting buffer (25 mM Tris/HCl, 192 mM glycine and 25% methanol in Milli-Q water) for 20 min to remove SDS. The protein was trans-blotted to nitrocellulose membrane using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Four sheets of 7×8 cm Whatman 3 MM filter paper and a sheet of the same size of reinforced nitrocellulose membrane (Sigma-Aldrich) were pre-wetted in trans-blotting buffer for 2 min. The gel sandwich was piled up in the Transfer Cell by placing two sheets of filter paper on the bottom, covering them with a sheet of nitrocellulose membrane, carefully placing the gel on top of the membrane and then covering the gel with another two sheets of filter paper. The gel was trans-blotted at 10 V for 60 min. The membrane was blocked in 1% casein/PBS solution for 1 h, and then transferred to primary Ab solution (1 μg/ml in 0.5% casein/PBS solution), incubating at room temperature for 1 h on a rocking shaker. The membrane was washed 3 times with PBST, 10 min per wash, and then HRP-conjugated secondary Ab solution (1:1,000 dilution in 0.5% casein/PBS) was added to the membrane, incubating for 1 h. After three washes as before, the bound second Ab was detected by ECL reagent, as described in section 2.6.3.

2.7 Recombinant protein expression by mammalian expression systems

2.7.1 Transient transfection of COS-7 cells by DEAE/dextran

One day prior to transfection, COS-7 cells were trypsinised, reseeded at a density of 6×10^6 cells per T75 (75 cm^2 growth surface) tissue culture flask and cultured in a humidified 5% CO_2 incubator at 37ºC. When the cells were ready for transfection, a transfection mix for a T75 flask was prepared, in 15 ml DMEM medium, by diluting 114 µg endotoxin-free plasmid DNA, 0.1 mM chloroquine and 600 µg/ml DEAE/dextran. Washing the cell layer twice with sterile PBS to remove any trace of FCS, the DNA/DEAE complex was added to the cells and the flask was returned to the incubator for 3.5 h. The supernatant in the flask was removed, the cell layer was washed once with sterile PBS, and then the cells were shocked by adding 10 ml of 10% DMSO/PBS for 2 min. Discarding the DMSO solution and replacing it with 15 ml of complete DMEM medium, the flask was returned to the incubator and the cells were cultured overnight. The growth medium was then replaced with serum-free medium. Three days later, the supernatant containing fusion protein was harvested.

2.7.2 Transient transfection of COS-7 cells by electroporation

Full-length cDNA in expression vectors were electroporated into COS-7 cells using a Gene Pulser Xcell electroporation system (Bio-Rad). One day before transfection, the COS-7 cells were split and reseeded in new tissue culture flasks, as described in section 2.7.1. On the day of transfection, the cells were trypsinized, pelleted and washed with RPMI 1640 medium once. The cells were then resuspended

in RPMI 1640 medium without any supplements, counted and adjusted to a cell density of 1×10^7 cells/ml. For a single transfection, 100 µl of cell suspension were mixed with 10 µg of plasmid DNA and the mixture transferred to a 0.2 cm cuvette (Bio-Rad, UK). Electrophoration was performed using the pre-set protocol for COS-7 cells: 110 V for 20 msec. After electrophoration, 500 µl pre-warmed complete DMEM medium were added to the cuvette and the whole of the cell suspension was transferred to a Universal. The volume of cell suspension was brought up to 5 ml with complete DMEM medium and the viable cells were counted. Cells (2×10^3) were then grown on 13 mm round coverslips (VRW, UK) in 24-well plates for 48 h.

2.8 Immunostaining of transfected COS-7 cells on cover slides

In brief, after tipping off the supernatant, the cells were washed once with 1 ml per well of PBS and then fixed by addition of 200 µl per well of 4% paraformaldehyde/PBS solution for 30 min at room temperature on a rocking shaker. After washing the cells three times, the cells were permeablized with 1% Triton X-100 (Sigma-Aldrich) in PBS (200 µl per well) for 15 min. After washing the wells three times again, primary mAb solution (1µg/ml in 1% BSA/PBS, 200 µl/well) was added and incubated for 1 h at room temperature. After 3 washes, a second antibody, HRP-conjugated goat anti-mouse IgG antibody in 1:1,000 dilution in 1% BSA/PBS, 200 µl/well, was added and incubated for 1 h. The HRP activity was detected by an AEC substrate kit (BD Pharmingen, UK). The AEC substrate solution was made up by diluting a drop of AEC chromogen in 1 ml AEC buffer, and then brought up to 10 ml with Milli-Q water. After 3 washes, 200 µl/well AEC solution were added to the plate, incubating for 5-20 min. Washing the plate 3 times again, Mayer's haematoxylin solution (Sigma-Aldrich), 500 µl/well, was added to the plate and

incubated for 2-5 min. The plate was washed with normal tap water until the water was colourless and then washed three times with Scott's water (20 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l $NaHCO_3$). The coverslips were picked out from the wells with fine-tip forceps, dried on tissue paper and then mounted onto glass slides with the cell-side facing down. The coverslips were sealed with nail vanish and labelled, and the results were observed under a microscope.

2.9 Affinity chromatography

Affinity chromatography was used to deplete bovine IgG from FBS, purify recombinant Ig protein from tissue culture supernatant or monoclonal antibodies from hybridoma culture supernatants. Protein G columns (1 ml or 5 ml, GE Healthcare, Amersham, UK) were used as immunoaffinity tools and connected to an EP-1 Econo peristaltic pump, (Bio-Rad) to continuously inject fluid.

Prior to the samples being injected into the column, they were aliquoted in 50 ml Falcon tubes and then centrifuged at 3,000 *g* for 30 min to remove large particles. The sample was then filtered through a Nalgene filtration unit with a 0.45 µm pore size membrane (Thermal Scientific) to remove smaller particles. Adjusting flow rate to 1 ml/min for a 1 ml column or 5 ml/min for a 5 ml column, the column was washed with a 20-column volume of PBS. The sample was then pumped through the column. To remove any trace of non-binding contamination, the column was washed again with a 20-column volume of PBS. Meanwhile, the collecting tubes were prepared by adding 100 µl of 1 M Tris/HCl (pH 9), to neutralize the elution buffer to pH 7. The protein was then eluted from the column with 0.1 M glycine, pH 2.7, and 1 ml of elution fractions were collected in the prepared tubes. The protein fractions were measured with a NanoDrop (Thermal Scientific). If the total eluted protein was

beyond the column maximum binding capacity, the flowthrough went through the column again.

For bovine IgG depletion, 500 ml serum normally went through a 5 ml protein G column twice to remove as much IgG as possible. The flowthrough was then filtered through a filtration unit, aliquoted and stored at -20ºC. For purification of recombinant Ig protein or antibody, the elution fractions containing protein were pooled and dialyzed in a Slide-A-Lyzer dialysis cassette (Thermal Scientific) against PBS on a magnetic stirer. After changing the PBS three times, the protein in the dialyzer was harvested, filtered through a 0.22 μm syringe filter, aliquoted and stored at 4ºC for short term, or -20ºC for long term, storage.

2.10 Flow cytometric analysis

For immunostaining, cells were washed twice with cold PBS and resuspended in 1% BSA/PBS. After counting, the cell density was adjusted to 2×10^6 cells/ml. Cells were plated at 100 μl/well in a U-bottomed 96-well plate and incubated on ice for 30 min to allow BSA blocking any non-specific binding. The cells were then pelleted by centrifugation at 450 *g* for 1 min, and 50 μl per well of primary mAb in 1% BSA/PBS at appropriate dilution added to the wells, mixed thoroughly on a plate shaker and incubated on ice for 1 h. The cells were pelleted as before and washed twice with 200 μl 1% BSA/PBS. Secondary Abs (conjugated with different fluorescent dyes) were added to wells (50 μl/well), incubating on ice for 1 h. The cells were pelleted again and the cell pellet washed twice as before. The cells in each well were then resupended in 100 μl 1% BSA/PBS, transferred to a FACs tube and topped up to 200 μl with 1% BSA/PBS. The cells were analysed by flow cytometry

using a FacsCalibur instrument (BD Becton Dickinson). The resulting data were analysed with FlowJo software.

Chapter 3. Identification and characterisation of the chicken Tim family molecules.

3.1 Introduction

Effector Th1/Th2 cells orchestrate immune responses against a wide variety of pathogenic microorganisms, and regulate/suppress immune responses both to control autoimmunity and to adjust the magnitude and persistence of responses. As described in Chapter 1, when naïve $CD4^+$ T cells encounter antigen-activated APCs, they differentiate into Th1 and Th2 cells driven by different transcription factors, and produce different pattern of cytokines. Th1 cells produce IFN-γ, which is modulated by T-bet. IL-4, -5 and -13 are Th2 cell signature cytokines which are regulated by GATA-3. The Th2 locus, including the genes for IL-4, -5 and -13, lies on human chromosome 5q23-35 and a homologous chromosomal region of the mouse genome 11B1.3. Both regions are strongly associated with asthma susceptibility, allergy and autoimmunity (Loots et al., 2000; Yokouchi et al., 2000; Walley et al., 2001). An intensive study of this chromosomal region in a mouse model of human asthma identified a T cell and airway phenotype regulator (Tapr) locus, which controls the development of airway hyperreactivity and T cell production of IL-4 and IL-13. The Tim family genes lie in this locus (McIntire et al., 2001). As described in Chapter 1, the murine Tim family has 8 genes, Tim1-4 encode proteins but Tim5-8 are pseudogenes. The human Tim family has 3 members, Tim1, Tim3 and Tim4, which are homologous to murine Tim1, Tim3 and Tim4 respectively. The Tim molecules are type I proteins with a common structure, containing IgV, mucin and transmembrane domains and a cytoplasmic tail. Human Tim1 and Tim3 are

preferentially expressed on Th2 and Th1 cells respectively, and play crucial roles in regulation of Th1 and Th2 cell proliferation. Human Tim4 is specifically expressed on APCs, especially on macrophages, and plays a role in enhancement of phagocytosis of dead cells by macrophages.

The chicken genome sequence was released in 2004 in public databases, simplifying identification of new genes. To identify the chicken Tim (chTim) genes, the human and murine Tim amino acid sequences were used to search the chicken genome in ENSEMBL. Based on information obtained from the blast searches, the chTim cDNAs were then amplified by RT-PCR. To study their roles in the immune system, recombinant chicken Tim proteins were expressed in mammalian expression systems.

3.2 Materials and Methods

3.2.1 Cloning chicken Tim family genes by PCR

The amino acid sequences of human Tim1 (NP_036338), Tim4 (NP_001140198) or Tim3 (NP_116171) were used as sequences in Blast searches (http://www.ensembl.org/Gallus_gallus/blastview) against the chicken EST and genome databases (v.38) in ENSEMBL. The blasting hits matched two clusters of overlapping chicken EST sequences, which are homologous to human Tim1 and Tim4 respectively. More confidence was gained by further analysis of the chromosomal region, where several marker genes surrounding the genes of interest are conserved in syntenical location between chicken and human. Therefore, primers were designed based on the longest EST sequences and synthesised by Sigma. The primer sequences are as listed in Table 3.1.

Primer	Sequence (5'-3')
Tim1-F1	AAT AAG AGT GTT CCT CAT C
Tim1-R1	AAG TTT TTA ATT AGA ATT GTA GC
Tim1-F2	ATG TCT TCT CAT TTC TTC C
Tim1-R2	TTA ATT AGA ATT GTA GCT TTT ATT TG
Tim4-F1	AGC CAA AAT GTC CCA CTT T
Tim4-R1	ACA CAG ATC CCA GAA ATA CTA CTG
Tim4-F2	ATG TCC CAC TTT GTG TTG TTT C
Tim4-R2	TCA CAG CAC AAA AAG GTT GT

Table 3.1. Primers used to amplify chicken Tim family cDNAs.

cDNA generated from splenic total mRNA from a 6-week-old line 7_2 bird, as described in section 2.5.1, was used as PCR template to clone the Tim cDNA, as described in section 2.5.2. A nested PCR strategy was used to clone each cDNA with touch-up or touch-down settings to increase the efficiency of primer binding.

The chicken Tim1 cDNA was amplified using the primer pair Tim1-F1/R1. After denaturation of the cDNA template at 94°C for 3 min, a touch-up PCR was carried out with cycling conditions of 94°C for 1 min, 50°C for 30 s and 72°C for 90 s for 3 cycles; 94°C for 1 min, 52°C for 30 s and 72°C for 90 s for 3 cycles; 94°C for 1 min, 54°C for 30 s and 72°C for 90 s for 30 cycles. The first-round PCR product was then used as template for amplification with a nested primer pair, Tim1-F2/R2. The reaction involved denaturation at 94°C for 3 min, followed by 34 cycles of 94°C for 1 min, 50°C for 30 s and 72°C for 2 min.

For the chicken Tim4 cDNA, Tim4-F1/R1 primers were used in the first-round amplification, followed by a touch-down PCR with cycling conditions of 94°C for 3 min; 94°C for 1 min, 60°C for 30 s and 72°C for 2 min for 3 cycles; 94°C for 1 min, 58°C for 30 s and 72°C for 2 min for 3 cycles; 94°C for 1 min, 57°C for 30 s and 72°C for 2 min for 30 cycles. Again the first-round PCR product was used as template for amplification with a nested primer pair, Tim4-F2/R2. The PCR conditions were as follows: 94°C for 3 min; 94°C for 1 min, 60°C for 30 s and 72°C for 2.5 min for 3 cycles; 94°C for 1 min, 61°C for 30 s and 72°C for 2.5 min for 30 cycles.

Nested PCR products were electrophoresed through 1% agarose gels (section 2.5.3). The bright and clear bands were excised from the gel and extracted using a gel extraction kit (section 2.5.4). Subsequently, the PCR amplicons were ligated into the vector pGEM-T Easy (Promega) and transformed into *E. coli* JM109 competent cells (sections 2.5.5 and 2.5.6). Plasmids isolated from positive colonies were sequenced (section 2.5.8).

3.2.2 Construction of expression plasmids

The full-length chTim1, chTim4S and chTim4L amino acid sequences were input into SMART software (http://smart.embl-heidelberg.de/) to predict their signal peptide, IgV, mucin, transmembrane and cytoplasmic domains. Based on this information, primers with restriction enzyme sites (Table 3.2) were designed to amplify different domains, which in turn were subcloned into a relevant expression vector for expression of fusion proteins using different expression systems, as summarised in Table 3.3.

Primer	Sequence (5'-3')
Tim1-IgVF	***GCT AGC*** ATG TCT TCT CAT TTC TTC
Tim1-IgVR	***AGA TCT*** TCA ACC ACC ACC TGG AGG TT
Tim1-mucinF	***GCT AGC*** AGA GCT AGG GTC TCT ACT
Tim1-mucinR	***AGA TCT*** TTT TCT GAA TAC TGC TGA CT
Tim4S-IgVF	***GCT AGC*** ATG TCC CAC TTT GTG TT
Tim4S-IgVR	***AGA TCT*** AGC ACC ACC AGC TGA ATG TT
Tim4S-mucinF	***GCT AGC*** GAA GCA CCT CCA TTG ATG
Tim4S-mucinR	***AGA TCT*** GGA AAG GAA AGT TTC ATC CC
Tim4L-HF	***GCT AGC*** GAA GTA TTT GAT GAG ACA
Tim4L-HR	***AGA TCT*** GAT GCA GTG TTC ATT GC

Table 3.2. Primers used to generate expression constructs. The bold italic nucleotides show the *Nhe*I or *Bgl*II restriction enzyme sites.

Gene	Domain	Fragment	Primer	Expression vector	Protein	Protein expression method
Tim1	IgV	M_1-E_{130}	Tim1-IgVF/R	pKW06-Ig	chTim1-IgV-Ig	Transient COS-7
	Mucin	R_{131}-K_{207}	Tim1-mucinF/R	Signal pKW06-Ig	chTim1-mucin-Ig	Transient COS-7
	Extracellular	M_1-K_{207}	Tim1-IgVF /-mucinR	pKW06-Ig	chTim1-Ig	Stable CHO
		M_1-K_{207}	Tim1-IgVF /-mucinR	pKW06-His6	chTim1-His6	Stable CHO
Tim4S	IgV	M_1-L_{130}	Tim4S-IgVF/R	pKW06-Ig	chTim4S-IgV-Ig	Transient COS-7
	Mucin	E_{131}-P_{209}	Tim4S-mucinF/R	Signal pKW06-Ig	chTim4S-mucin-Ig	Transient COS-7
	Extracellular	M_1-P_{209}	Tim4S-IgVF /-mucinR	pKW06-Ig	chTim4S-Ig	Stable CHO
		M_1-P_{209}	Tim4S-IgVF /-mucinR	pKW06-His6	chTim4S-His6	Stable CHO
Tim4L	IgV	M_1-L_{316}	Tim4S-IgVF/R	pKW06-Ig	chTim4L-2IgV-Ig	Transient COS-7
	Extracellular	M_1-P_{485}	Tim4S-IgVF /-mucinR	pKW06-Ig	chTim4L-Ig	Transient COS-7
	Hinge	E_{131}-S_{207}	Tim4L-HF/R	Signal pKW06-Ig	chTim4S-h-Ig	Stable CHO

Table 3.3. Summary of expression of recombinant chTim proteins. The chTim domains to be expressed were cloned as different fragments by PCR with the listed primer pairs (Table 3.2). The resulting PCR products were then subcloned into relevant expression vectors. The tagged-fusion proteins were eventually expressed from stably transfected CHO or transiently transfected COS-7 cells.

Different domains of the chicken Tim molecules were amplified by PCR and *Nhe*I and *Bgl*II restriction enzyme cleavage sites were introduced to the ends of the PCR products (section 2.5.2). The PCR products were then purified from agarose gels (sections 2.5.3 and 2.5.4). The purified DNA fragments were cloned into pGEM-T Easy and the plasmids transformed into *E.coli* JM109 competent cells (sections 2.5.5 and 2.5.6). Inserts were confirmed by DNA sequencing (section 2.5.8) and then excised from the plasmid with *Nhe*I and *Bgl*II (section 2.5.9). The digested

DNA fragments were then ligated into mammalian expression vectors (section 2.5.10).

For chTim1, chTim4S and chTim4L, the IgV domains (two repeat IgV domains for chTim4L) with gene-specific signal peptides were inserted into the pKW06-Ig vector (Figure 2.1); the mucin domains of chTim1 and chTim4S, as well as the hinge part of chTim4L (between two IgV domains) were ligated into the Signal pKW06-Ig vector (Figure 2.3). The extracellular domains of chTim1, chTim4S and chTim4L, containing gene-specific signal peptides, IgV and mucin domains, were ligated into pKW06-Ig for all three genes or into the pKW06-His6 vector (Figure 2.2) for chTim1 and chTim4S.

Again, the resultant expression plasmids were then transformed into *E.coli* JM109 competent cells. Cloned plasmids were prepared from the resulting colonies and sequenced to confirm the plasmid constructs. DNA plasmids were then prepared with large-scale endotoxin-free Maxiprep kits (QIAGEN) (section 2.5.11).

3.2.3. Establishment of stably transfected CHO cell cultures

CHO cells were cultured in complete Ham's F12 medium, as described in section 2.3.2, split and reseeded in T25 tissue culture flasks with 1×10^6 cells/flask. After overnight culture, CHO cells were transfected with plasmid DNA (or untransfected as controls) using Lipofectamine 2000 (Invitrogen), as per the manufacturer's instructions. Briefly, two 1.5 ml Eppendorf tubes were filled with 250 µl of Ham's F12 medium without any supplements. Plasmid DNA (10 µg) was added to one tube and the lipofectamine reagent (25 µl) to the other. After incubation for 5 min at room temperature, the solutions were combined and further incubated

for 25 min. The DNA complex was then added to the CHO cells and mixed by swirling the flasks. One day after transfection, the medium was changed for selective medium, complete Ham's F12 medium plus 20 μg/ml puromycin. The medium was changed every two days to remove dead cells until any untransfected CHO control cells were all dead and the transfected cells appeared to be growing in colonies (foci with hundreds of cells). Ten to twelve days after the cells were placed in selective media, the number of foci in transfected flasks was estimated by counting the clones on a 1 cm^2 growing surface.

To screen for high-yield protein clones, the cells were split, counted and reseeded into 96-well flat tissue culture plates at approximately 1 cell/well. After 6 days growth at 37ºC, 5% CO_2, the number of cell clones in each well was counted. Early stage scoring of the clones has the benefit of avoiding multiple clones merging into a large single clone in some wells. After 10 days growth in selective medium, when the diameter of cell clones was about 1 mm, the screening process was performed. CHO cell transfectants expressing hIgG Fc-tagged fusion proteins were screened by capture ELISA, as described in section 2.6.2, except without titration of the samples. CHO clones expressing His6-tagged proteins were screened by dot-blot analysis, as described in section 2.6.3. The selected positive clones were split and cultured in 24-well plates at 37ºC, 5% CO_2. Multiple clones were selected to grow up, in case of false positives in the first round screening. When the cells were fully confluent in 24-well plates, the supernatants were subjected to a second round of screening. The highly-ranked clones were frozen down at this stage, and several clones chosen to grow up in T25 tissue culture flasks. When the cells were fully confluent, the supernatants were re-tested. The highest yielding clone was chosen to

grow up firstly in T75 flasks, then in T150 flasks. Stable CHO clones producing high yields of protein were established.

3.2.4. Large-scale protein expression by miniPERM culture

A miniPERM is a bioreactor designed for the culture of hybridomas (Falkenberg, 1998) and other cells, e.g. stable CHO transfectants (Shutt et al., 1997), at high densities ("high density culture"). The culture apparatus is composed of two modules: a 40 ml disposable cell culture and protein production chamber (the 'production module') and a 550 ml medium reservoir (the 'nutrition module'). The two modules are separated from each other by a dialysis membrane (molecular weight cut off 12.5 kDa) allowing passage of low molecular mass nutrients and metabolites. To start the culture, briefly, cells were trypsinized from two T175 flasks at full confluence, pelleted by centrifugation at 1,000 *g* for 5 min and the cell pellet resuspended in 40 ml Ham's F12 medium plus 5% Ig-depleted FBS (section 2.9) and 15 µg/ml puromycin. The cell suspension was injected into the production module through ports in a silicone rubber bung and the nutrition module was topped up with 400 ml Ham's F12 medium plus 5% normal FBS. The bioreactor was rolled at 0.2 rolls/min on a roller apparatus at 37ºC, 5% CO_2 for 4 days. The sample was harvested from the production module from injection port and the cells were separated by centrifugation at 1,000 *g* for 5 min. Keeping the supernatant for protein, the cells were resupended in Ham's F12 medium plus 5% Ig-depleted FBS and 15 µg/ml puromycin and the cell density adjusted to 5×10^6 cells/ml. The cell suspension (40 ml) was then injected into the production module for the next round of culture. The medium in the nutrition module was changed every two harvests.

3.2.5. Purification of human IgG Fc-tagged fusion protein

Ig fusion proteins in supernatants from the miniPERM cultures were purified on a 1 ml protein G column, as described in section 2.9.

3.2.6. Purification of His6-tagged fusion protein

The supernatants from the miniPERM cultures, containing His6-tagged fusion proteins, were aliquoted in 50 ml Falcon tubes, centrifuged at 3,000 *g* for 30 min, and then filtered through a filtration unit with a 0.4 μm pore size membrane (Thermal Scientific). Ni-NTA (50% nickel bead slurry, GE Healthcare) slurry (20 ml) was transferred to a 50 ml Falcon tube, the beads separated from the liquid by centrifugation at 2,000 *g* for 5 min, and washed twice with 30 ml PBS. The resin was then added to the supernatant and stirred on a magnetic stirrer overnight at 4ºC. The nickel resin-bearing protein was allowed to settle by gravity. The supernatant was carefully removed. The beads were resuspened in 20 ml PBS, and then transferred to a 20 ml gravity-flow column (Bio-Rad). After the beads were allowed to settle, the column was washed with 20 ml PBS, and then 50 ml 20 mM imidazole/PBS (pH 8.0). The protein bound to the column was dissociated with the addition of 15 ml 150 mM imidazole/PBS (pH 8.0). The elution was collected in 0.5 ml fractions in 1.5 ml Eppendorf tubes. The amount of protein in each fraction was measured using a Nanodrop. The fractions containing the protein were then pooled together. Any imidazole in the protein solution was removed by dialysis against PBS (section 2.9). The protein solution was then filtered through a 0.22 μm syringe filter.

3.3 Results

3.3.1 Identification of the chicken Tim family genes

The amino acid (aa) sequences of human Tim1, Tim4 or Tim3 were used as sequences in Blast searches against the chicken EST and genome databases in ENSEMBL. Two clusters of overlapping chicken EST sequences were found which were highly homologous with human Tim1 and Tim4 respectively (Figure 3.1) and were located on chicken chromosome 13. Further analysis of the sequences around this locus showed conserved synteny with mammals, in that chicken Tim1 and Tim4 genes lie next to each other between ITK and SGCD (Figure 3.2). The murine Tim locus lies on chromosome 11 with several intervening pseudogenes, and the human Tim family genes are next to each other on chromosome 5. Analysis of the available genomes of other avian species showed that the turkey has the same number of Tim family molecules flanked by the same genes as the chicken, but they are located on chromosome 15. Similarly to the human Tim family, the zebra finch Tim family has three members, Tim1, Tim3 and Tim4, on chromosome 13, but unlike human, Tim3 lies between ITK and Med7.

3.3.2 Amplification of the chicken Tim family genes

Based on the longest ESTs identified in the Blast searches, primers were designed to clone cDNAs encoding the predicted chicken Tim1 and Tim4 proteins, as described in section 3.2.1, using splenic cDNA as a template. An approximate 1 kb band was amplified for chicken Tim1 (Figure 3.3A). Interestingly, two bands of 1.1 kb and 1.5 kb were amplified for chicken Tim4 (Figure 3.3B). The purified PCR products from the agarose gel were subcloned by ligation into a TA-cloning vector

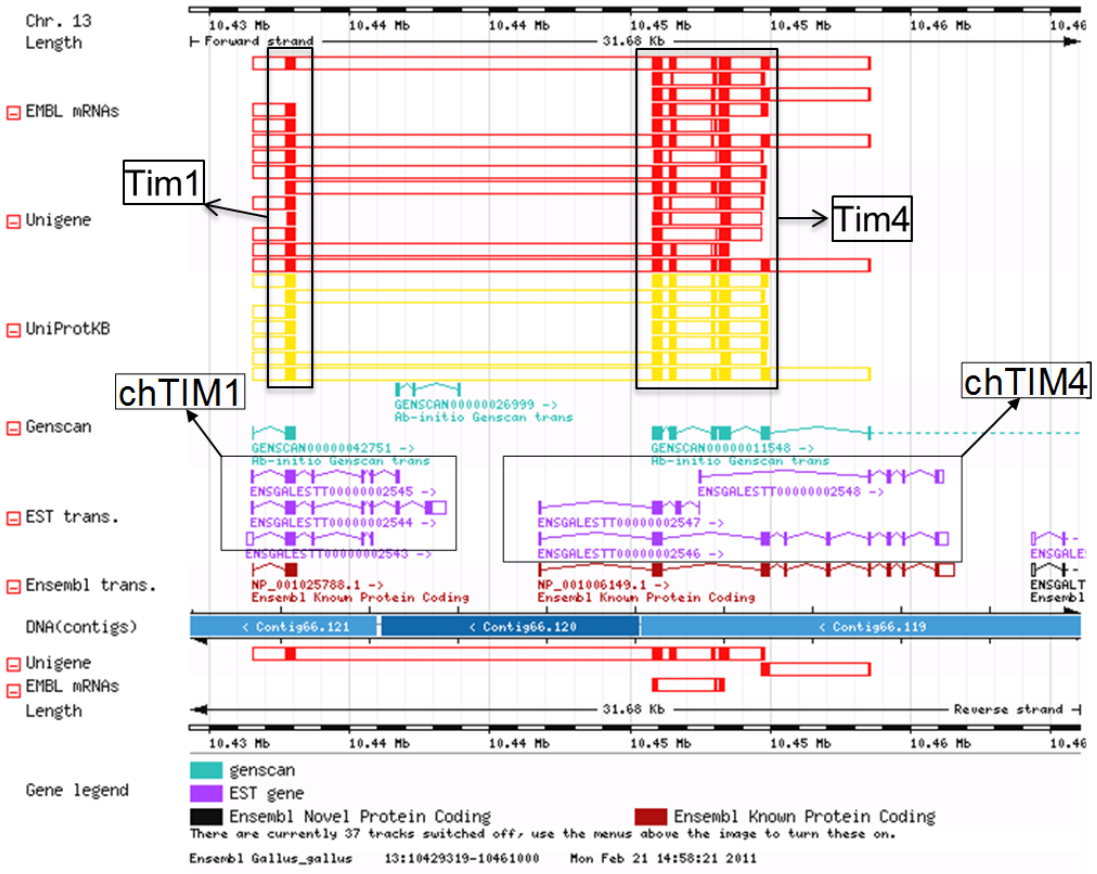
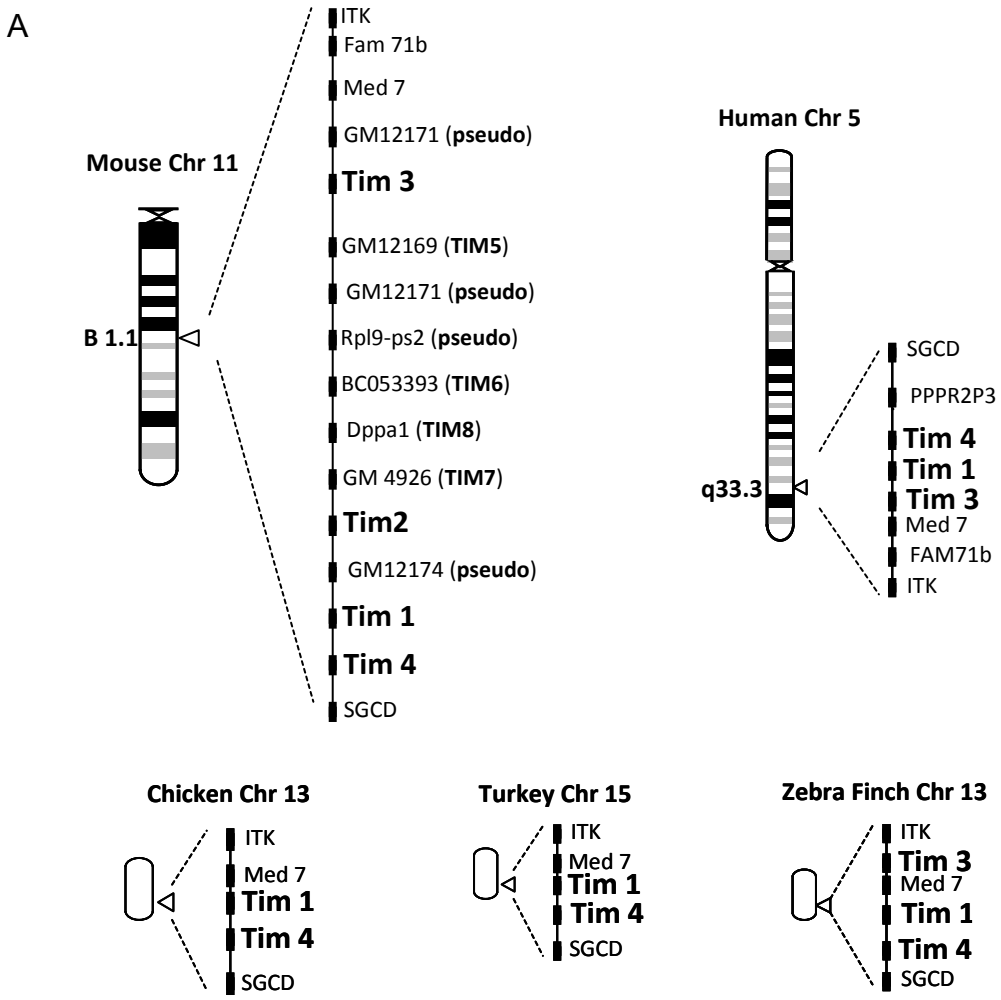


Figure 3.1. Schematic diagram of annotation of and evidence for the chicken Tim family members from ENSEMBL, release version 38 (April 2006). ENSEMBL's annotations are shown in dark red. Two clusters of chicken Tim EST sequences are boxed and labelled. The supportive evidence, mammalian Tim1 and Tim4 mRNAs from different databases, are boxed and shown in different colours, including mRNA from EMBL and Unigene in red, UniProtkb in yellow and Genscan annotation in green. The Genscan programme-predicted chicken Tim genes are shown in light blue.


A
Mouse Chr 11
B 1.1
ITK
Fam 71b
Med 7
GM12171 (pseudo)
Tim 3
GM12169 (TIM5)
GM12171 (pseudo)
Rpl9-ps2 (pseudo)
BC053393 (TIM6)
Dppa1 (TIM8)
GM 4926 (TIM7)
Tim2
GM12174 (pseudo)
Tim 1
Tim 4
SGCD
Human Chr 5
q33.3
SGCD
PPPR2P3
Tim 4
Tim 1
Tim 3
Med 7
FAM71b
ITK
Chicken Chr 13
ITK
Med 7
Tim 1
Tim 4
SGCD
Turkey Chr 15
ITK
Med 7
Tim 1
Tim 4
SGCD
Zebra Finch Chr 13
ITK
Tim 3
Med 7
Tim 1
Tim 4
SGCD

B

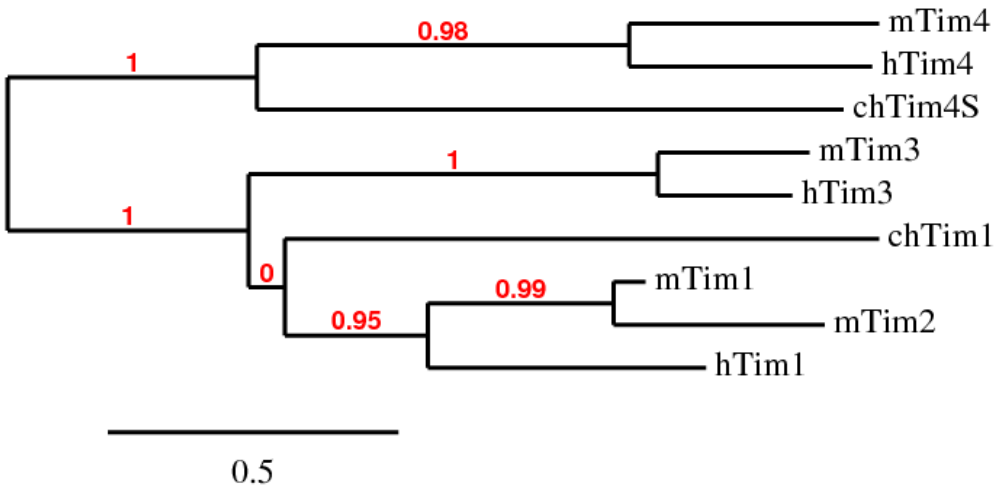

0.98
1
1
1
0
0.99
0.95
mTim4
hTim4
chTim4S
mTim3
hTim3
chTim1
mTim1
mTim2
hTim1
0.5

Figure 3.2. Tim family genes. (A). Genomic location and arrangement of Tim family genes in different species. The primary data for mouse, human and chicken are derived from the NCBI database, for turkey from the pre-ENSEMBL database and zebra finch from the ENSEMBL database. (B). Phylogenetic tree of human (h), mouse (m) and chicken (ch) Tim family molecules. Full-length Tim protein sequences were aligned using ClustalW2. Sequence similarity is proportional to the length of the line that connects two sequences, as estimated by phylogeny.fr program (http://www.phylogeny.fr/version2_cgi/). The protein sequences of hTim1 (NP036338), hTim3 (NP_116171), hTim4 (NP_001140198), mTim1 (AAL35775), mTim2 (NP_001154827), mTim3 (NP_599011) and mTim4 (NP_848874) were obtained from NCBI database. The numbers next to each node, in red, represent a measure of support for the node, where 1 represents maximal support. Scale bar indicates the number of aa changes per site.

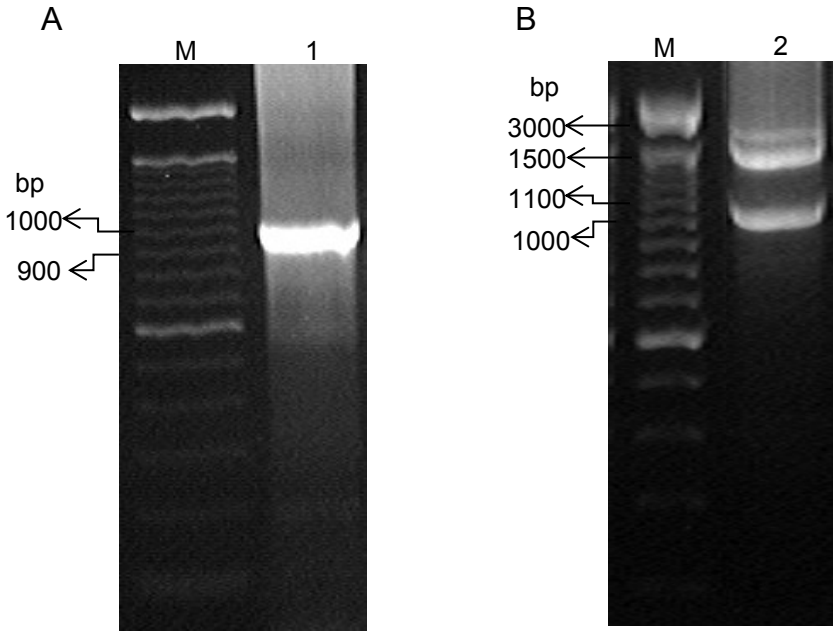


Figure 3.3. Amplification of chicken Tim cDNAs by RT-PCR. Splenic cDNA from a line 7_2 bird was used as template. (A). Lane 1 is PCR product (chicken Tim1) amplified with primers Tim1-F2/R2. (B). Lane 2 is PCR product (chicken Tim4) amplified with primers Tim4-F2/R2. M = 100 bp DNA Ladder (Invitrogen)

and transformation into competent cells. Ten cloned plasmids from each transformation were then sequenced. Sequencing confirmed that the chicken Tim4 molecule has two variants, described as long and short isoforms, which will be explained further below.

3.3.3 Structure of the chicken Tim1 molecule

The phylogenetic tree showed that chicken Tim1 is homologous to human and mouse Tim1 (Figure 3.2B). The sequence of the chicken Tim1 (chTim1) cDNA encodes an open reading frame of 275 aa (Figure 3.4). SMART (http://smart. emblheidelberg.de/), SignalP (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM (www.cbs.dtu. dk/services/ TMHMM-2.0/) programs were used to predict the structure of chTim1. ChTim1 is a type I protein with a signal peptide consisting of 21 aa and a transmembrane domain spanning aa 208–230. The extracellular domain of chTim1 is 176 aa in length and contains two distinctive domains. Similarly to human and mouse Tim1, the NH_2 terminus of the chTim1 extracellular domain (aa 22-127) is homologous to Ig superfamily (IgSF) variable domains, such as those of TCR and Ig (Barclay et al., 1993), and a similar structure to those seen in mammalian Tim family members, where it is named an IgV domain. Like other IgSF molecules, the chTim1 IgV domain contains two typical cysteine residues (Cys37 and Cys110) to presumably form an intramolecular disulfide bond, as demonstrated by the crystal structure of the murine Tim1 protein (Santiago et al., 2007b). ChTim1 also possesses four additional cysteines in the IgV domain, as seen in the Ig domain of murine Tim1, where the crystal structure indicated that they form two additional disulphide bonds, resulting in a novel structure different from the Ig domain of other IgSF members (Santiago et al., 2007b). This novel structure forms a pocket, where a

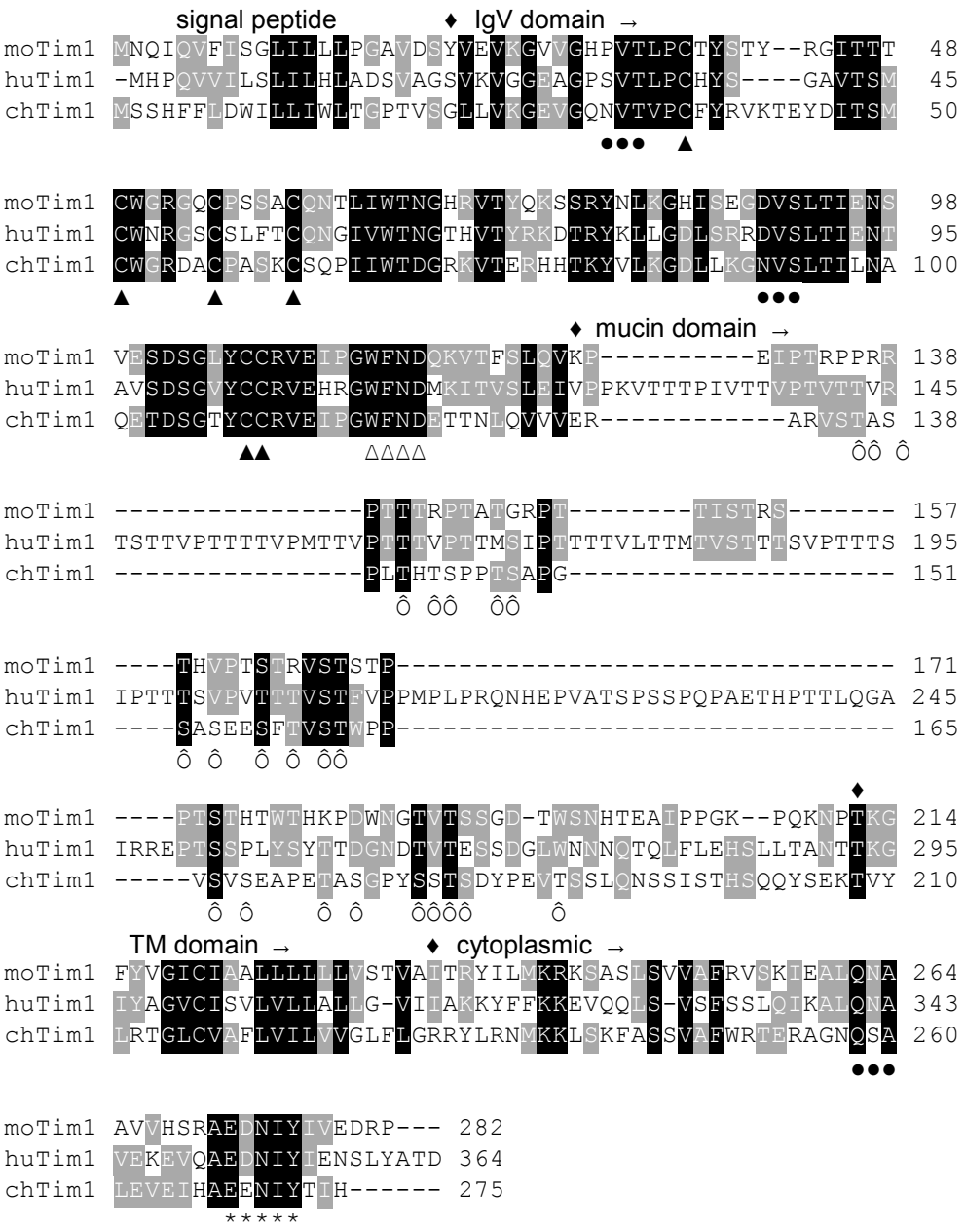


Figure 3.4. Alignment of chicken Tim1 with human (hu) (NP036338) and mouse (mo) Tim1 (AAL35775.1). Identical residues are shaded in black, less homologous residues in grey. The 6 conserved cysteines are identified with filled triangles, and empty triangles indicate PS binding sites. Asterisks indicate tyrosine kinase phosphorylation sites. N-linked glycosylation sites in chicken Tim1 are highlighted by solid dots and O-linked glycosylation sites labelled as ô. Filled diamonds label the start-points of each domain.

phosphatidylserine (PS) binding site is present, including a PS binding motif (WFND residues). The second distinctive structure in the chTim1 extracellular domain is a mucin domain from aa 130 to 207, containing high numbers of serine, threonine and proline residues (T/S/P-rich), among which 23 potential O-linked glycosylation sites are predicted by NetOGlyc (http://www. cbs.dtu.dk/services /NetOGlyc/), as shown in Figure 3.4. ChTim1 also has three potential N-linked glycosylation sites, two (N32 and N92) in the IgV domain and one (N257) in the cytoplasmic region, predicted by NetNGlyc (http://www.cbs.dtu.dk/services/ NetNGlyc/) (Figure 3.4). The cytoplasmic domain of the chTim1 consists of 45 aa and contains two tyrosines, one of which (Y272) is present in a conserved motif (NED/ENIY). This conserved tyrosine in murine Tim1 is a phosphorylation site and initiates downstream signalling pathways (de Souza et al., 2005). Multiple sequence alignment showed that chicken and mouse Tim1 have 33% aa identity and chicken and human Tim1 32%. The chTim1 IgV domain is highly homologous and shares 47.7% identity with that of human Tim1, and 53.2% identity with that of mouse Tim1.

3.3.4 Structure of the chicken Tim4S molecule

The phylogenetic tree also showed that chTim4S is homologous to mammalian Tim4 (Figure 3.2B). However, unlike mammals, two forms of chicken Tim4, a short isoform (chTim4S) and a long isoform (chTim4L), were initially identified. ChTim4S encodes a 359 aa membrane-bound type I protein with a signal peptide (aa 1-21) and a transmembrane domain spanning aa 300-320 (Figure 3.5). In the extracellular domain of chTim4S, an NH_2-terminal IgV domain (aa 22-130) contains a PS-binding motif (WFND) and six conserved cysteines (Figure 3.5). The first and last cysteines presumably form an intramolecular disulphide bond as in

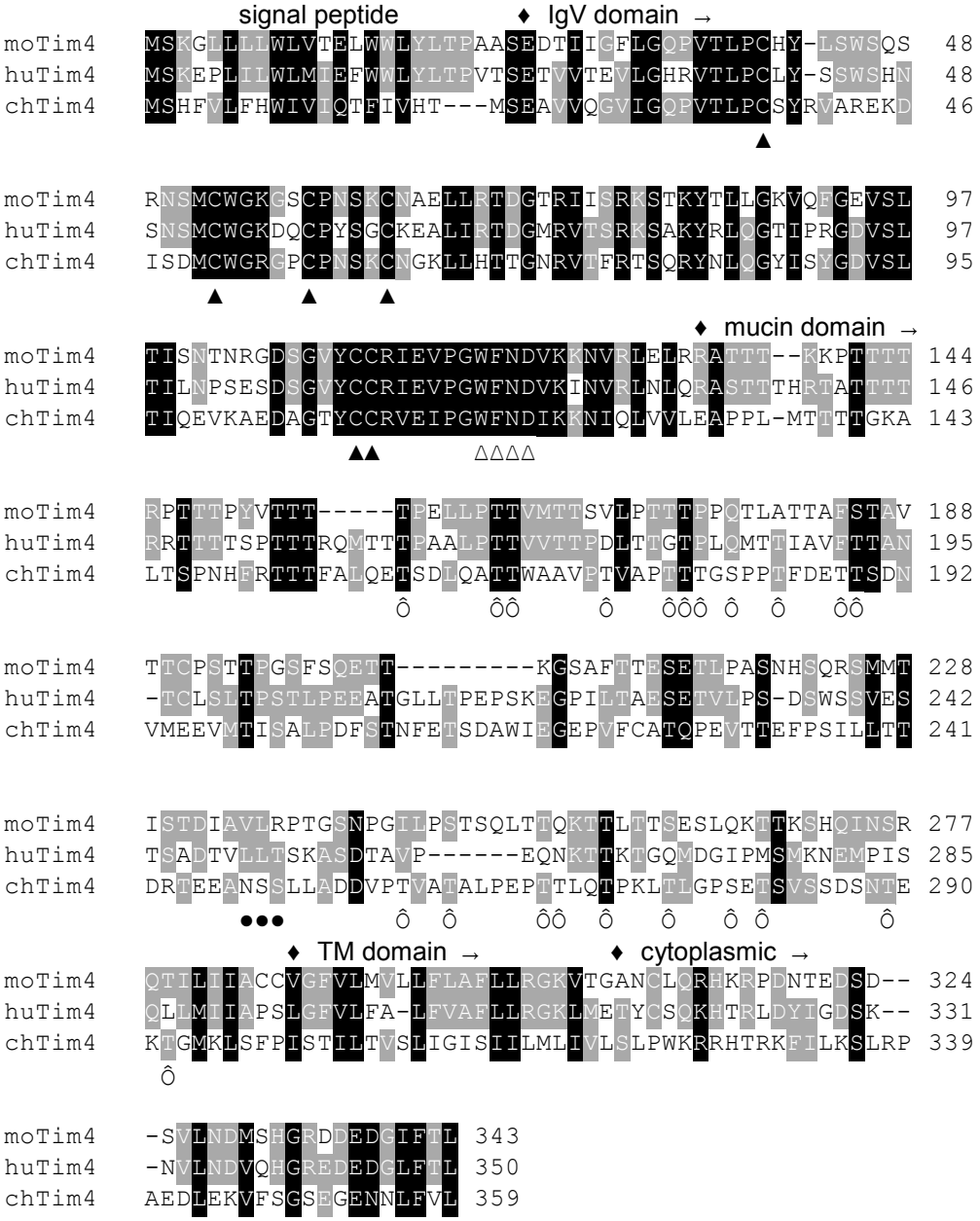


Figure 3.5. Alignment of chicken Tim4S (chTim4) with mouse (mo) Tim4 (NP848874.3) and human (hu) Tim4 (NP001140198.1). Identical residues are shaded in black, less homologous residues in grey. Filled triangles emphasized the 6 conserved cysteines and empty triangles indicate PS binding sites. Potential N-linked glycosylation sites in chTim4 are highlighted by solid dots and O-linked glycosylation sites labelled as ô. Filled diamonds label the start-points of each domain.

other IgSF Ig domains, and four additional cysteines presumably form two additional disulphide bonds to create a cleft where PS binds, as shown in the IgV domain of murine Tim4 (Santiago et al., 2007a). Following the IgV domain in the extracellular domain, a mucin domain spans aa 131-299 and also contains a high number of threonine, serine and proline residues with 20 predicted O-linked glycosylation sites (Figure 3.5). A single potential N-linked glycosylation site (N248) is found in the mucin domain. The cytoplasmic tail (aa 321-359) of chTim4S lacks tyrosine residues, suggesting, like its mammalian equivalents, that chTim4S does not have a signal transduction role. The highly homologous IgV domain has 50.5% aa identity with human and 51.4% aa identity with mouse Tim4 (Figure 3.5).

3.3.5 Structure of the chicken Tim4L molecule

ChTim4L is a novel isoform of the chicken Tim4 molecule, not yet described in mammals. The structure of the chTim4L protein was predicted by SMART, as shown in Figure 3.6. The chTim4L cDNA encodes a membrane-bound type I protein of 545 aa, containing a signal peptide (aa 1-21), a mucin (aa 316-487) domain, a transmembrane (aa 488-510) domain and a short cytoplasmic tail (aa 511-545) without any significant peptide motif. Interestingly, chTim4L has two almost identical IgV domains (aa 22-130 and 208-316) linked by a short polypeptide chain (aa 131-207). Alignment of the putative aa sequences of chTim4L and chTim4S indicated that chTim4L and chTim4S share identical signal peptide, mucin and cytoplasmic domains, but there are aa differences between them in the IgV and transmembrane domains (Figure 3.6). For example, T23, T97, A128 in the IgV domain and L509 in the mucin domain of chTim4L correspond to A23, I97, V128 (in the IgV domain) and P313 (in the mucin domain) of chTim4S.

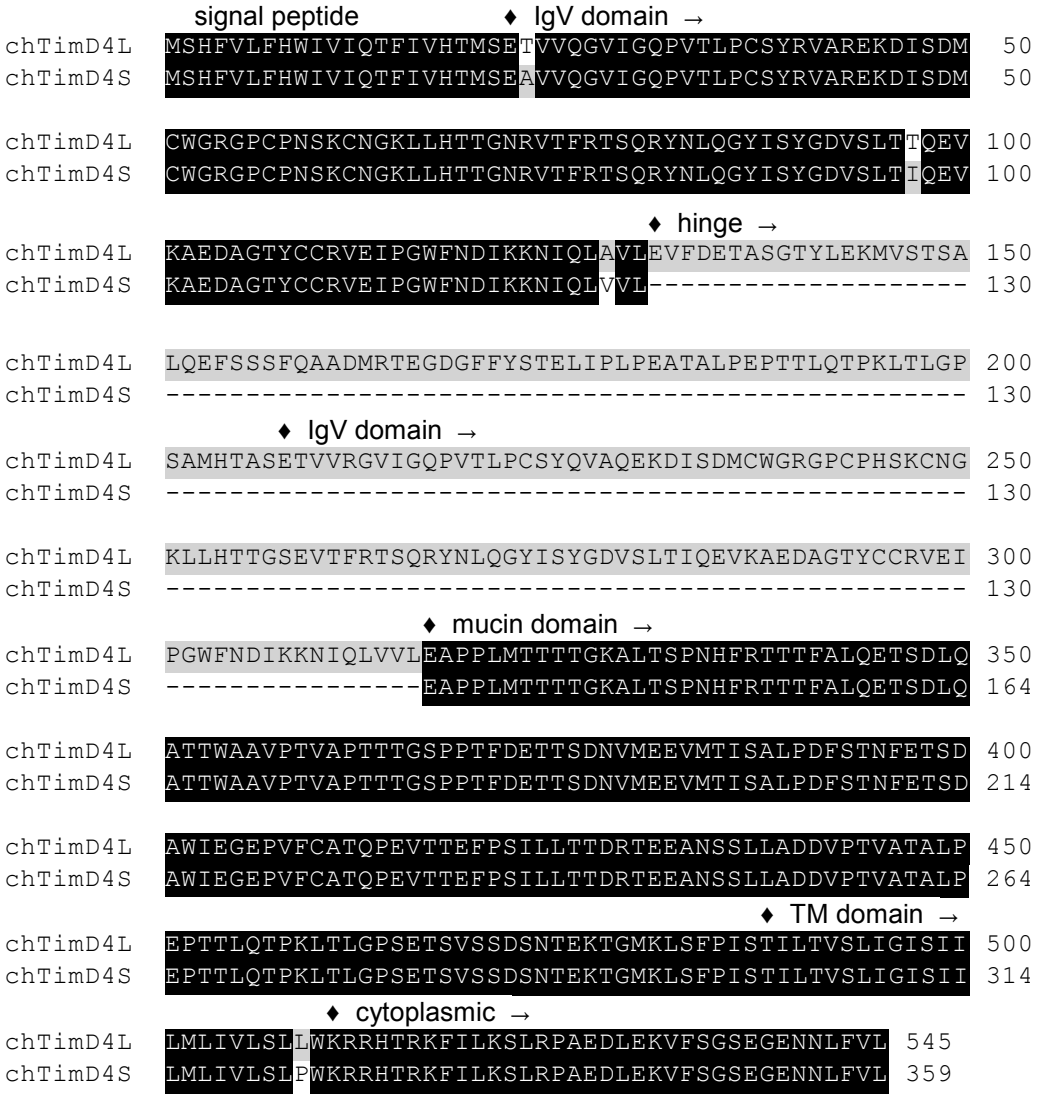


Figure 3.6. Alignment of chTim4S and chTim4L. Both of their cDNAs were amplified from splenic cDNA from a line7_2 bird. Filled diamonds label the start-points of each domain. The domains are labelled above sequences. Identical residues are shaded in black, less homologous residues in grey. Dashes show gaps.

3.3.6 Polymorphisms in chTim4S and chTim4L

To determine if similar patterns of polymorphisms of chTim4S and chTim4L are consistent in other chicken strains, their cDNAs were amplified again from a 6-week-old J line bird. Total RNA was isolated from: 1) spleen, 2) rchCD40L-stimulated adherent splenocytes and 3) rchCD40L-stimulated suspension splenocytes, to determine if chTim4S and chTim4L have different expression patterns in different cell populations. The rchCD40L-stimulated splenocytes were prepared as follows: splenocytes were isolated from the rest of spleen tissue (section 2.3.5.1) and cultured with rchCD40L fusion protein for 72 h (section 2.3.5.4); the resulting adherent and the suspension splenocytes were harvested separately for isolation of total RNA (section 2.4). After reverse transcription of total RNA into cDNA (section 2.5.1) as PCR templates, chTim4S and chTim4L cDNAs were amplified from them with the primer pair Tim4-F1/R1, as described in section 3.2.1.

As shown in Figure 3.7A, a bright band of 1.1 kb was amplified from the cDNA from spleen tissue. Interestingly, amplification from cDNA from the rchCD40L-stimulated adherent and suspension cells both resulted in two different sizes of bands. The former had two larger bands (which will be further described in section 3.3.7), whereas the latter had two bands of different intensity and obviously different molecular size (Figure 3.7A). A nested PCR with the primer pair Tim4-F2/R2 (section 3.2.1) was also used to determine if the two bands from the cDNA from rchCD40L-stimulated suspension cells are chTim4 cDNAs. As shown in Figure 3.7B, each nested PCR reaction resulted in a clear bright band of a similar size to their templates. The PCR products, including the bright band amplified from the spleen tissue and both nested PCR bands (Figure 3.7B), were then subcloned into

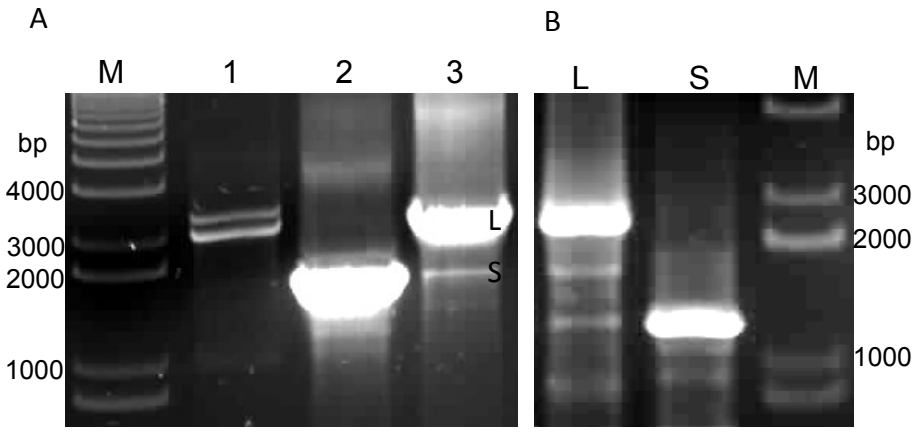
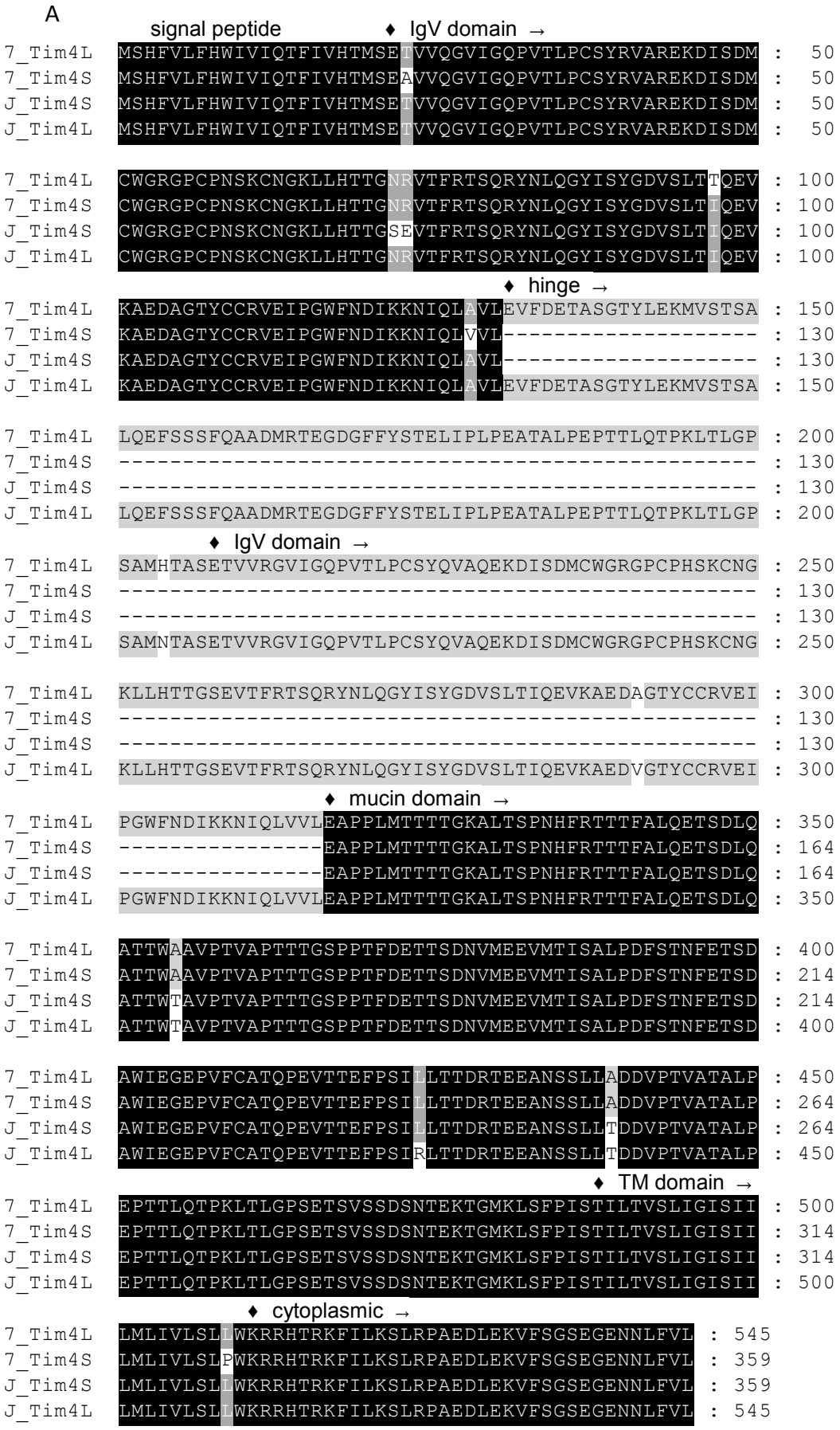


Figure 3.7. Amplification of chTim4 cDNA from a J line bird. (A). cDNAs from rchCD40L-stimulated adherent splenic cells (lane 1), spleen tissue (lane 2) and rchCD40L-stimulated suspension splenocytes (lane 3) were used as PCR templates with primer pair Tim4-F1/R1. (B). Nested-PCR products in lane L and S were amplified using the large (L) and small (S) bands in lane 3 in (A) as templates, with primer pair Tim4-F2/R2. M = TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen).

pGEM-T Easy for sequencing. The sequencing results indicated that the PCR products from spleen tissue and the small band from the nested PCR were chTim4S, whereas the large band from the nested PCR was chTim4L.

The putative aa sequences of chTim4L and chTim4S isolated from the J line bird were compared with those sequences cloned from the original line 7_2 bird, as shown in Figure 3.8A. There were six aa differences in the chTim4L molecules between two line (T97I, H204N, A291V, A355T, L423R and A438T), and seven in chTim4S (A23T, N24S, R25E, V127A, A135T, A252T and P323L). It remains to be determined if these aa changes would affect their biological functions, but none of these polymorphisms occur in known important aa residues, such as the cysteines involved in intramolecular disulphate bonds or the PS binding sites.


A
signal peptide
♦ IgV domain →
7_Tim4L MSHFVLFHWIVIQTFIVHTMSETVVQGVIGQPVTLPCSYRVAREKDISDM : 50
7_Tim4S MSHFVLFHWIVIQTFIVHTMSEAVVQGVIGQPVTLPCSYRVAREKDISDM : 50
J_Tim4S MSHFVLFHWIVIQTFIVHTMSETVVQGVIGQPVTLPCSYRVAREKDISDM : 50
J_Tim4L MSHFVLFHWIVIQTFIVHTMSETVVQGVIGQPVTLPCSYRVAREKDISDM : 50
7_Tim4L CWGRGPCPNSKCNGKLLHTTGNRVTFRTSQRYNLQGYISYGDVSLTTQEV : 100
7_Tim4S CWGRGPCPNSKCNGKLLHTTGNRVTFRTSQRYNLQGYISYGDVSLTIQEV : 100
J_Tim4S CWGRGPCPNSKCNGKLLHTTGSEVTFRTSQRYNLQGYISYGDVSLTIQEV : 100
J_Tim4L CWGRGPCPNSKCNGKLLHTTGNRVTFRTSQRYNLQGYISYGDVSLTIQEV : 100
♦ hinge →
7_Tim4L KAEDAGTYCCRVEIPGWFNDIKKNIQLAVLEVFDETASGTYLEKMVSTSA : 150
7_Tim4S KAEDAGTYCCRVEIPGWFNDIKKNIQLVVL---------- : 130
J_Tim4S KAEDAGTYCCRVEIPGWFNDIKKNIQLAVL---------- : 130
J_Tim4L KAEDAGTYCCRVEIPGWFNDIKKNIQLAVLEVFDETASGTYLEKMVSTSA : 150
7_Tim4L LQEFSSSFQAADMRTEGDGFFYSTELIPLPEATALPEPTTLQTPKLTLGP : 200
7_Tim4S ---------- : 130
J_Tim4S ---------- : 130
J_Tim4L LQEFSSSFQAADMRTEGDGFFYSTELIPLPEATALPEPTTLQTPKLTLGP : 200
♦ IgV domain →
7_Tim4L SAMHTASETVVRGVIGQPVTLPCSYQVAQEKDISDMCWGRGPCPHSKCNG : 250
7_Tim4S ---------- : 130
J_Tim4S ---------- : 130
J_Tim4L SAMNTASETVVRGVIGQPVTLPCSYQVAQEKDISDMCWGRGPCPHSKCNG : 250
7_Tim4L KLLHTTGSEVTFRTSQRYNLQGYISYGDVSLTIQEVKAEDAGTYCCRVEI : 300
7_Tim4S ---------- : 130
J_Tim4S ---------- : 130
J_Tim4L KLLHTTGSEVTFRTSQRYNLQGYISYGDVSLTIQEVKAEDVGTYCCRVEI : 300
♦ mucin domain →
7_Tim4L PGWFNDIKKNIQLVVLEAPPLMTTTTGKALTSPNHFRTTTFALQETSDLQ : 350
7_Tim4S ----------EAPPLMTTTTGKALTSPNHFRTTTFALQETSDLQ : 164
J_Tim4S ----------EAPPLMTTTTGKALTSPNHFRTTTFALQETSDLQ : 164
J_Tim4L PGWFNDIKKNIQLVVLEAPPLMTTTTGKALTSPNHFRTTTFALQETSDLQ : 350
7_Tim4L ATTWAAVPTVAPTTTGSPPTFDETTSDNVMEEVMTISALPDFSTNFETSD : 400
7_Tim4S ATTWAAVPTVAPTTTGSPPTFDETTSDNVMEEVMTISALPDFSTNFETSD : 214
J_Tim4S ATTWTAVPTVAPTTTGSPPTFDETTSDNVMEEVMTISALPDFSTNFETSD : 214
J_Tim4L ATTWTAVPTVAPTTTGSPPTFDETTSDNVMEEVMTISALPDFSTNFETSD : 400
7_Tim4L AWIEGEPVFCATQPEVTTEFPSILLTTDRTEEANSSLLADDVPTVATALP : 450
7_Tim4S AWIEGEPVFCATQPEVTTEFPSILLTTDRTEEANSSLLADDVPTVATALP : 264
J_Tim4S AWIEGEPVFCATQPEVTTEFPSILLTTDRTEEANSSLLTDDVPTVATALP : 264
J_Tim4L AWIEGEPVFCATQPEVTTEFPSIRLTTDRTEEANSSLLTDDVPTVATALP : 450
♦ TM domain →
7_Tim4L EPTTLQTPKLTLGPSETSVSSDSNTEKTGMKLSFPISTILTVSLIGISII : 500
7_Tim4S EPTTLQTPKLTLGPSETSVSSDSNTEKTGMKLSFPISTILTVSLIGISII : 314
J_Tim4S EPTTLQTPKLTLGPSETSVSSDSNTEKTGMKLSFPISTILTVSLIGISII : 314
J_Tim4L EPTTLQTPKLTLGPSETSVSSDSNTEKTGMKLSFPISTILTVSLIGISII : 500
♦ cytoplasmic →
7_Tim4L LMLIVLSLLWKRRHTRKFILKSLRPAEDLEKVFSGSEGENNLFVL : 545
7_Tim4S LMLIVLSLPWKRRHTRKFILKSLRPAEDLEKVFSGSEGENNLFVL : 359
J_Tim4S LMLIVLSLLWKRRHTRKFILKSLRPAEDLEKVFSGSEGENNLFVL : 359
J_Tim4L LMLIVLSLLWKRRHTRKFILKSLRPAEDLEKVFSGSEGENNLFVL : 545

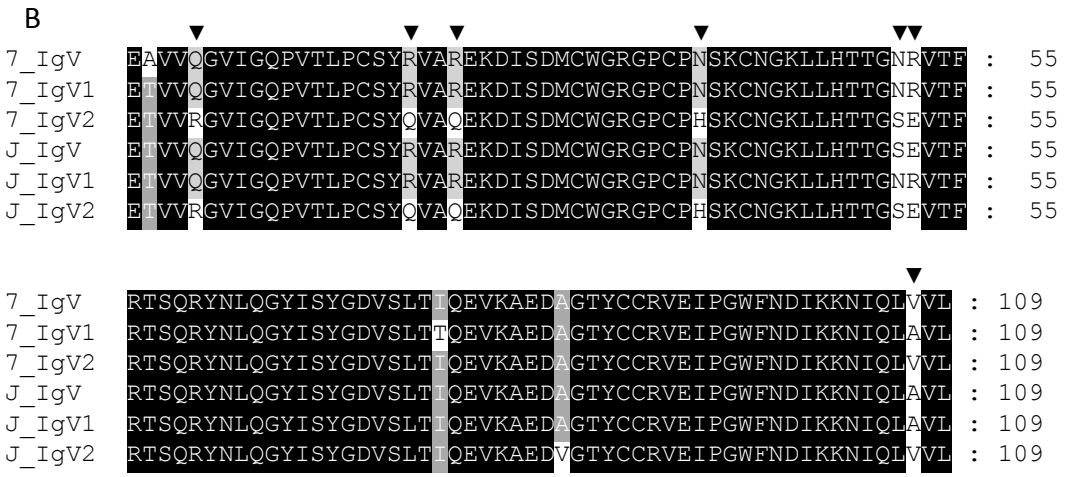


Figure 3.8. ChTim4S and chTim4L polymorphisms. (A). Alignments of chTim4S and chTim4L aa sequences from a J line and a line 7_2 bird. Filled diamonds label start-points of domains. The domains are labelled above sequences. (B). Alignment of IgV domain aa sequence; the two chTim4L IgV domains, named IgV1 and IgV2, were compared with the IgV domain of chTim4S. Identical residues are shown in black, less homologous residues in grey. Dashes show gaps. Filled triangles indicate the persistent aa variants between the IgV domains of chTim4S and chTim4L.

The aa sequences of the IgV domains in chTim4S and chTim4L from both lines of chickens were compared, as shown in Figure 3.8B. There were aa in the chTim4S IgV domain that were persistently different to those in either IgV domain of chTim4L. For example, in the line 7_2 bird, N72, R73 and A128 of chTim4S are identical to corresponding aa in IgV1 of chTim4L, but vary (S258, E259 and V314) in IgV2 of chTim4L. However, in the J line, S72, E73 and V128 in the IgV of chTim4S are different to N72, R73 and A128 in IgV1 of chTim4L, identical to S258 and E259 in IgV2 of chTim4L, but different to V314 (Figure 3.8B).

The polymorphisms between chTim4S and chTim4L are consistent in both lines of chicken. One possible explanation is that heterozygotes at the chTim4 locus

are common events in different lines of bird and chTim4S and chTim4L mRNAs may be randomly transcribed from different parental alleles.

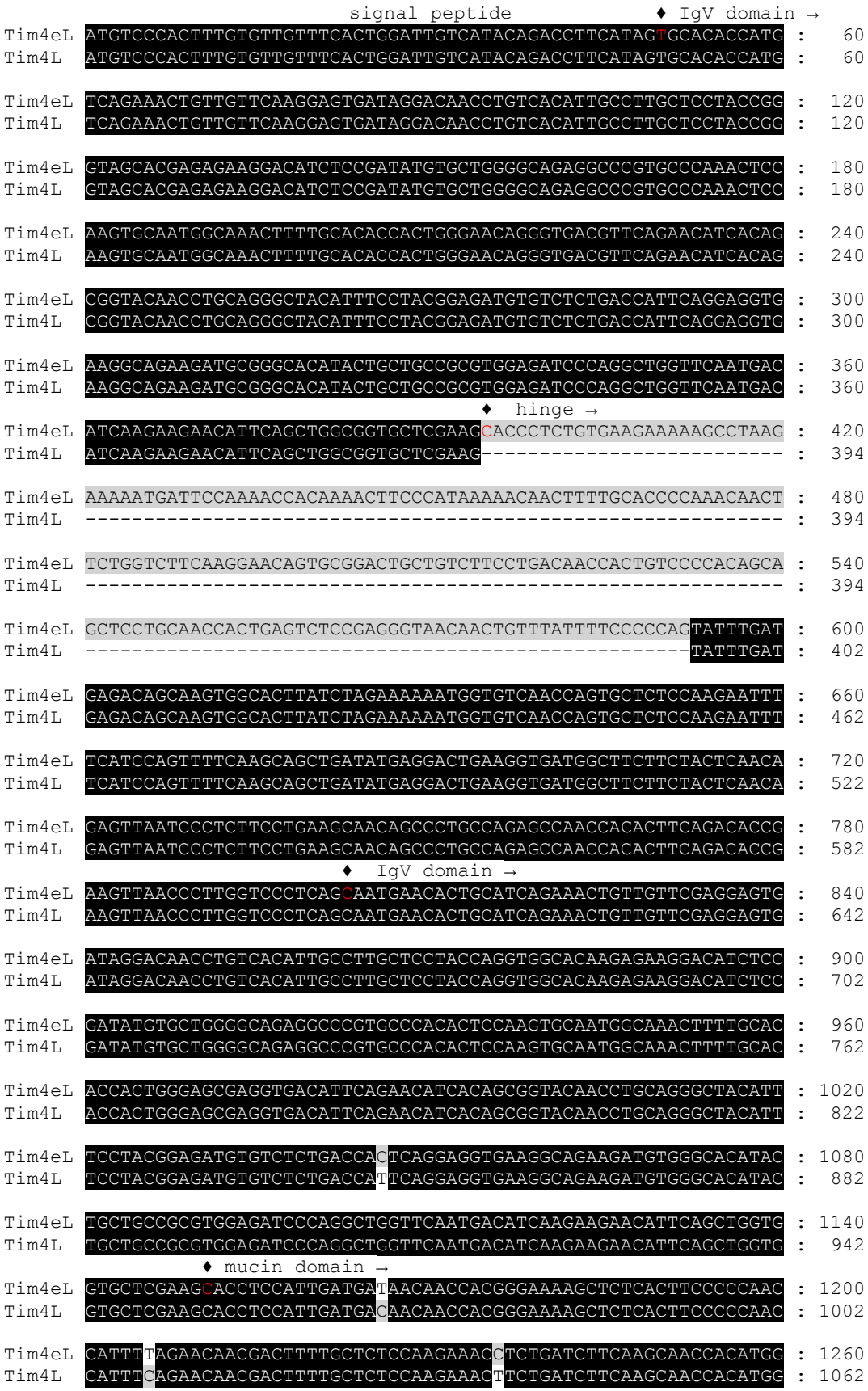
3.3.7 Identification of a new longer chTim4 isoform

As shown in Figure 3.7A, amplification of chTim4 from cDNA from rchCD40L-stimulated adherent splenocytes resulted in two large bands. Both of them (together) were then purified and subcloned in pGEM-T Easy. Eight cloned plasmids were sequenced. Seven of them contained chTim4L cDNA, but one plasmid contained cDNA even longer than that of chTim4L, as shown in Figure 3.9. This product was named chTim4-extra-long isoform (chTim4eL).

The chTim4eL cDNA has an open reading frame (ORF) of 1836 base-pairs (Figure 3.9). Sequence alignment of chTim4eL and chTim4L (Figure 3.9) indicated that chTim4eL is 198 bp longer than chTim4L in the hinge domain, and almost identical over the rest of the molecules. However, single nucleotide polymorphisms (SNPs) are also present between them (Figure 3.9).

3.3.8 Identification of a new shorter isoform of chTim4

Intensive analysis of the chTim4L cDNA sequences indicated that, as well as the sequences encoding the two repeated IgV domains, another two regions (545-605 bp and 738-798 bp) were also highly homologous. Further genomic analysis of these two regions indicated that they correspond to two different exons, exons 4 and 8 (the chTim4 gene structure will be further described below); interestingly, intronic sequences proximal to the 5' and 3' end of exons 4 and 8 also had high similarity. As shown in Figure 3.10, intron 3 (upstream of exon 4) is homologous to intron 7


signal peptide
♦ IgV domain →
Tim4eL ATGTCCCACTTTGTGTTGTTTCACTGGATTGTCATACAGACCTTCATAGTGCACACCATG : 60
Tim4L ATGTCCCACTTTGTGTTGTTTCACTGGATTGTCATACAGACCTTCATAGTGCACACCATG : 60
Tim4eL TCAGAAACTGTTGTTCAAGGAGTGATAGGACAACCTGTCACATTGCCTTGCTCCTACCGG : 120
Tim4L TCAGAAACTGTTGTTCAAGGAGTGATAGGACAACCTGTCACATTGCCTTGCTCCTACCGG : 120
Tim4eL GTAGCACGAGAGAAGGACATCTCCGATATGTGCTGGGGCAGAGGCCCGTGCCCAAACTCC : 180
Tim4L GTAGCACGAGAGAAGGACATCTCCGATATGTGCTGGGGCAGAGGCCCGTGCCCAAACTCC : 180
Tim4eL AAGTGCAATGGCAAACTTTTGCACACCACTGGGAACAGGGTGACGTTCAGAACATCACAG : 240
Tim4L AAGTGCAATGGCAAACTTTTGCACACCACTGGGAACAGGGTGACGTTCAGAACATCACAG : 240
Tim4eL CGGTACAACCTGCAGGGCTACATTTCCTACGGAGATGTGTCTCTGACCATTCAGGAGGTG : 300
Tim4L CGGTACAACCTGCAGGGCTACATTTCCTACGGAGATGTGTCTCTGACCATTCAGGAGGTG : 300
Tim4eL AAGGCAGAAGATGCGGGCACATACTGCTGCCGCGTGGAGATCCCAGGCTGGTTCAATGAC : 360
Tim4L AAGGCAGAAGATGCGGGCACATACTGCTGCCGCGTGGAGATCCCAGGCTGGTTCAATGAC : 360
♦ hinge →
Tim4eL ATCAAGAAGAACATTCAGCTGGCGGTGCTCGAAGCACCCTCTGTGAAGAAAAAGCCTAAG : 420
Tim4L ATCAAGAAGAACATTCAGCTGGCGGTGCTCGAAG---------- : 394
Tim4eL AAAAATGATTCCAAAACCACAAAACTTCCCATAAAAACAACTTTTGCACCCCAAACAACT : 480
Tim4L ---------- : 394
Tim4eL TCTGGTCTTCAAGGAACAGTGCGGACTGCTGTCTTCCTGACAACCACTGTCCCCACAGCA : 540
Tim4L ---------- : 394
Tim4eL GCTCCTGCAACCACTGAGTCTCCGAGGGTAACAACTGTTTATTTTCCCCCAGTATTTGAT : 600
Tim4L ----------TATTTGAT : 402
Tim4eL GAGACAGCAAGTGGCACTTATCTAGAAAAAATGGTGTCAACCAGTGCTCTCCAAGAATTT : 660
Tim4L GAGACAGCAAGTGGCACTTATCTAGAAAAAATGGTGTCAACCAGTGCTCTCCAAGAATTT : 462
Tim4eL TCATCCAGTTTTCAAGCAGCTGATATGAGGACTGAAGGTGATGGCTTCTTCTACTCAACA : 720
Tim4L TCATCCAGTTTTCAAGCAGCTGATATGAGGACTGAAGGTGATGGCTTCTTCTACTCAACA : 522
Tim4eL GAGTTAATCCCTCTTCCTGAAGCAACAGCCCTGCCAGAGCCAACCACACTTCAGACACCG : 780
Tim4L GAGTTAATCCCTCTTCCTGAAGCAACAGCCCTGCCAGAGCCAACCACACTTCAGACACCG : 582
♦ IgV domain →
Tim4eL AAGTTAACCCTTGGTCCCTCAGCAATGAACACTGCATCAGAAACTGTTGTTCGAGGAGTG : 840
Tim4L AAGTTAACCCTTGGTCCCTCAGCAATGAACACTGCATCAGAAACTGTTGTTCGAGGAGTG : 642
Tim4eL ATAGGACAACCTGTCACATTGCCTTGCTCCTACCAGGTGGCACAAGAGAAGGACATCTCC : 900
Tim4L ATAGGACAACCTGTCACATTGCCTTGCTCCTACCAGGTGGCACAAGAGAAGGACATCTCC : 702
Tim4eL GATATGTGCTGGGGCAGAGGCCCGTGCCCACACTCCAAGTGCAATGGCAAACTTTTGCAC : 960
Tim4L GATATGTGCTGGGGCAGAGGCCCGTGCCCACACTCCAAGTGCAATGGCAAACTTTTGCAC : 762
Tim4eL ACCACTGGGAGCGAGGTGACATTCAGAACATCACAGCGGTACAACCTGCAGGGCTACATT : 1020
Tim4L ACCACTGGGAGCGAGGTGACATTCAGAACATCACAGCGGTACAACCTGCAGGGCTACATT : 822
Tim4eL TCCTACGGAGATGTGTCTCTGACCACTCAGGAGGTGAAGGCAGAAGATGTGGGCACATAC : 1080
Tim4L TCCTACGGAGATGTGTCTCTGACCATTCAGGAGGTGAAGGCAGAAGATGTGGGCACATAC : 882
Tim4eL TGCTGCCGCGTGGAGATCCCAGGCTGGTTCAATGACATCAAGAAGAACATTCAGCTGGTG : 1140
Tim4L TGCTGCCGCGTGGAGATCCCAGGCTGGTTCAATGACATCAAGAAGAACATTCAGCTGGTG : 942
♦ mucin domain →
Tim4eL GTGCTCGAAGCACCTCCATTGATGATAACAACCACGGGAAAAGCTCTCACTTCCCCCAAC : 1200
Tim4L GTGCTCGAAGCACCTCCATTGATGACAACAACCACGGGAAAAGCTCTCACTTCCCCCAAC : 1002
Tim4eL CATTTTAGAACAACGACTTTTGCTCTCCAAGAAACCTCTGATCTTCAAGCAACCACATGG : 1260
Tim4L CATTTCAGAACAACGACTTTTGCTCTCCAAGAAACTTCTGATCTTCAAGCAACCACATGG : 1062

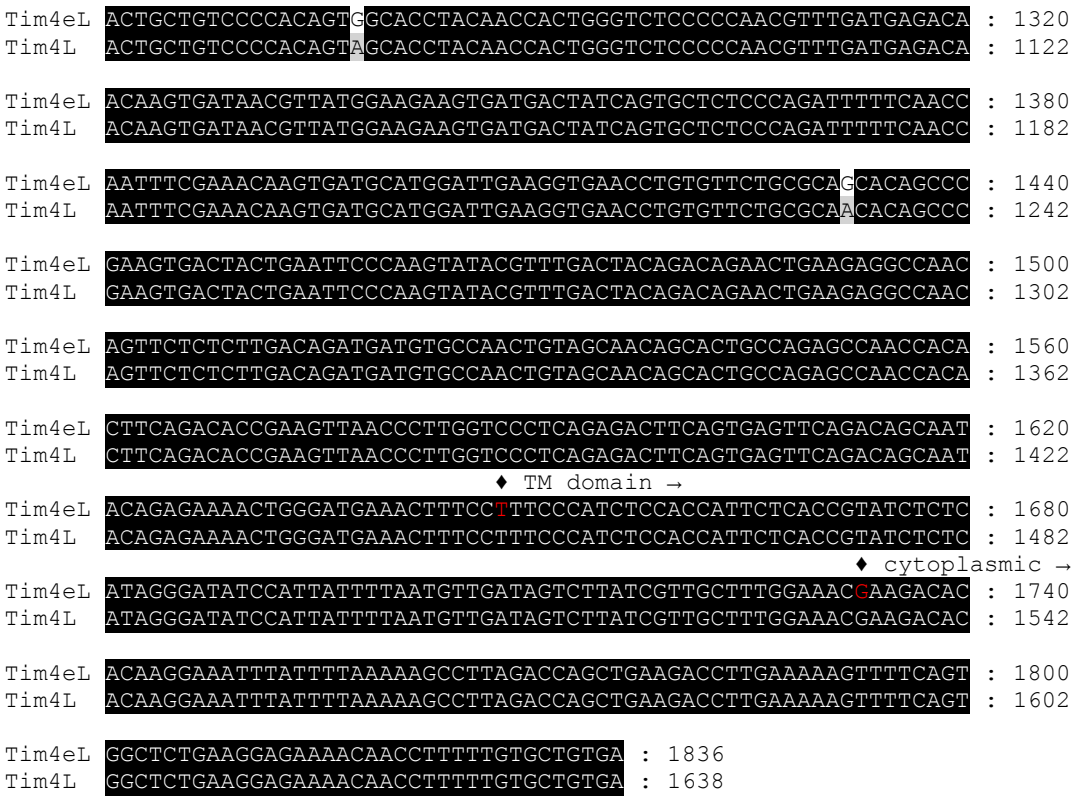


Figure 3.9. Alignment of chTim4L and chTim4eL sequences. Both chTim4L and chTim4eL were amplified from cDNA from the same J line bird. The start-points of domains are highlighted in red-coloured nucleotide residues and indicated by filled diamonds. The domains are labelled above the sequences. Identical residues are shown in black, less homologous residues in grey. Dashes show gaps.

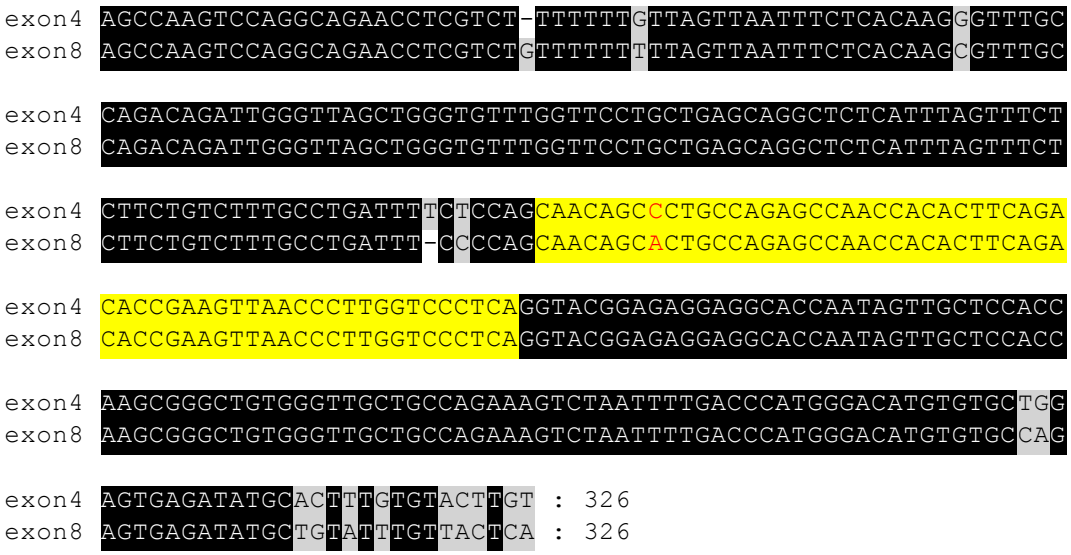


Figure 3.10. Alignments of exons 4 and 8 and intronic sequences proximal to them. Yellow-shaded nucleotides show exons 4 and 8 and red-coloured nucleotides show the variant. Black-shaded nucleotides are identical nucleotides in relevant intronic regions and grey-shaded nucleotides show differences. Dashes show gaps.

(upstream of exon 8) at their 3' ends; intron 4 (downstream of exon 4) is also homologous to intron 8 (downstream of exon 8) at their 5' ends, implying exons 4 and 8 may share an equal chance to be spliced during pre-mRNA splicing, because crucial factors for splicing are normally present in intronic regions proximal to a exon to be spliced, such as 5' splice donor sites in the downstream intron and 3' splice acceptor sites in upstream intron. The upstream intron also contains a branch sequence, the binding site for deposition of the spliceosome (a complex of snRNA and protein subunits) that removes introns from transcribed pre-mRNAs (Reed and Maniatis, 1988).

An attempt was then made to amplify this new potential variant of chTim4 by RT-PCR, as described in section 3.2.1. A cDNA template derived from the spleen of a 6-week-old J line bird was used as template in PCR amplification with the primer pair chTim-4F1/R1. As shown in Figure 3.11A, PCR resulted in a bright 1.2 kb band

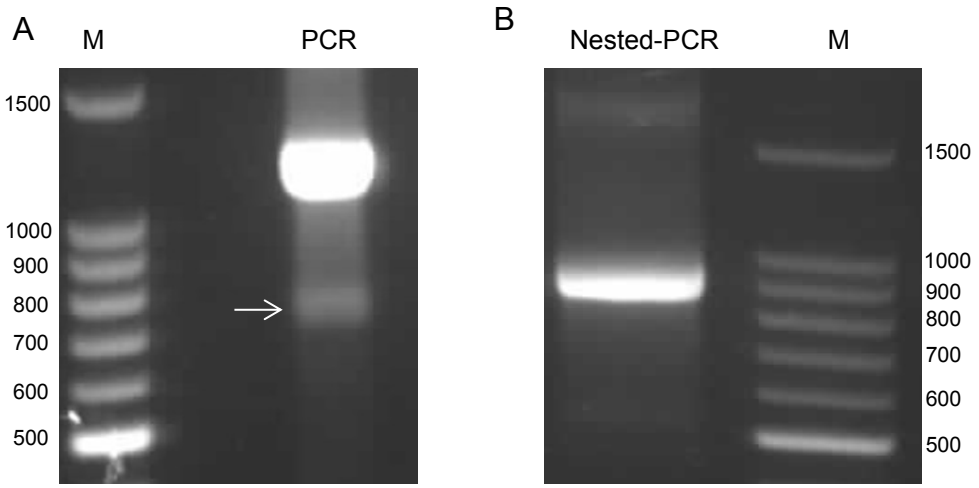
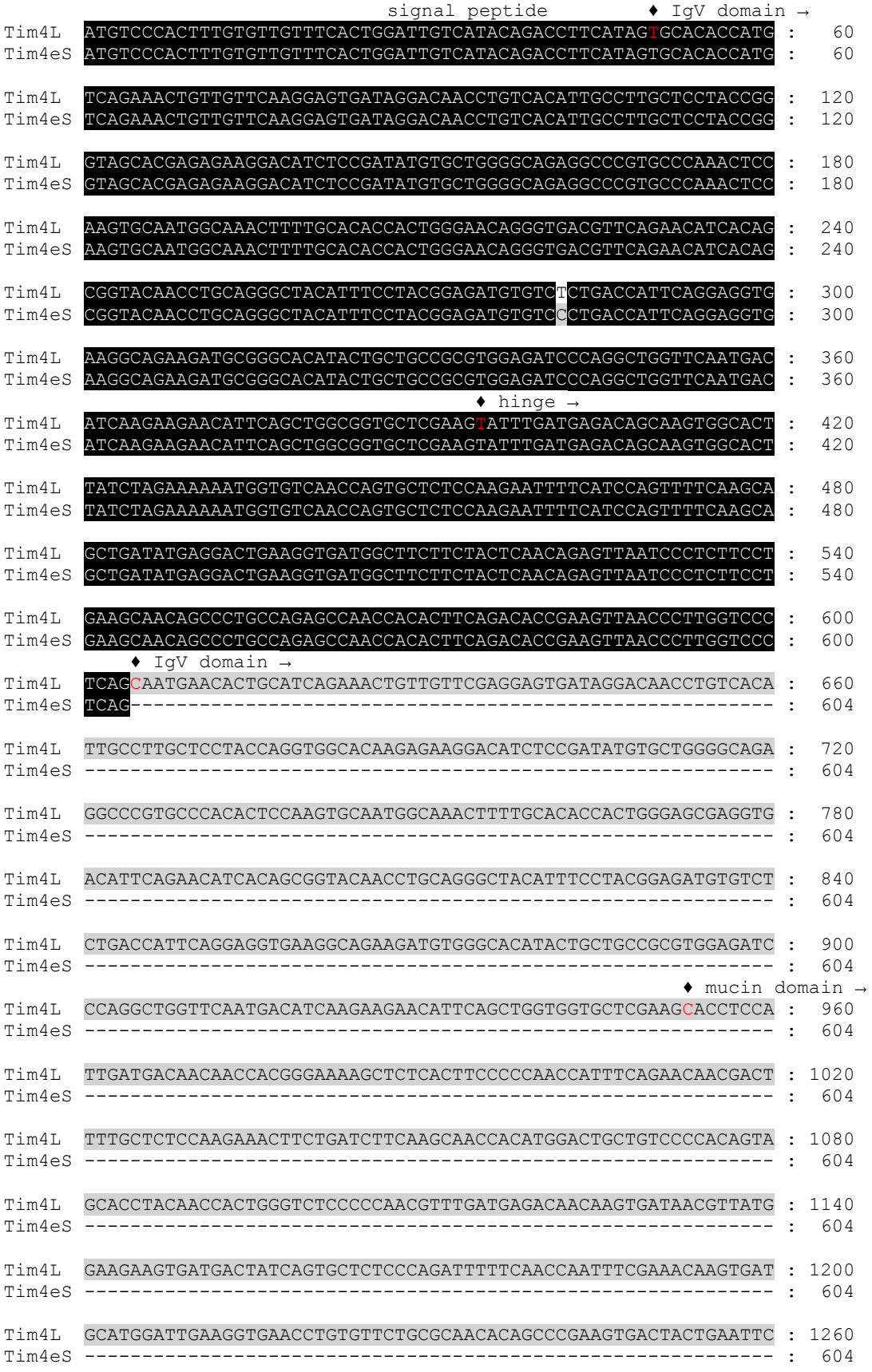


Figure 3.11. Amplification of an extra-short isoform of chTim4 by RT-PCR. (A). Splenic cDNA from a J line bird was used as template with primers chTim4F1/R1. (B). Nested PCR product amplified using the small band in (A, arrowed) as template with primers chTim4-F2/R2. M = 100 bp DNA ladder (Promega).

and a faint 800 bp band. The former had a similar molecular size to chTim4S, whereas the latter had never been seen before. The small band (800 bp) was then purified and used as template in a nested PCR with the primer pair chTim4-F2/R2 (section 3.2.1). As shown in Figure 3.11B, the nested PCR resulted in a bright band of similar molecular size to its template. This nested PCR product was then subcloned into pGEM-T Easy for sequencing. The sequencing results indicated that a new 846 bp cDNA was highly homologous to chTim4, and was therefore named chTim4-extra-short (chTim4eS) isoform. Sequence alignment of chTim4eS and chTim4L, as shown in Figure 3.12, indicated that both of them are almost identical in the signal peptide, IgV and transmembrane domains and cytoplasmic tails. However, chTim4eS has a very short mucin domain, but maintains a similar hinge region to that of chTim4L.


signal peptide
♦ IgV domain →
Tim4L ATGTCCCACTTTGTGTTGTTTCACTGGATTGTCATACAGACCTTCATAGTGCACACCATG : 60
Tim4eS ATGTCCCACTTTGTGTTGTTTCACTGGATTGTCATACAGACCTTCATAGTGCACACCATG : 60
Tim4L TCAGAAACTGTTGTTCAAGGAGTGATAGGACAACCTGTCACATTGCCTTGCTCCTACCGG : 120
Tim4eS TCAGAAACTGTTGTTCAAGGAGTGATAGGACAACCTGTCACATTGCCTTGCTCCTACCGG : 120
Tim4L GTAGCACGAGAGAAGGACATCTCCGATATGTGCTGGGGCAGAGGCCCGTGCCCAAACTCC : 180
Tim4eS GTAGCACGAGAGAAGGACATCTCCGATATGTGCTGGGGCAGAGGCCCGTGCCCAAACTCC : 180
Tim4L AAGTGCAATGGCAAACTTTTGCACACCACTGGGAACAGGGTGACGTTCAGAACATCACAG : 240
Tim4eS AAGTGCAATGGCAAACTTTTGCACACCACTGGGAACAGGGTGACGTTCAGAACATCACAG : 240
Tim4L CGGTACAACCTGCAGGGCTACATTTCCTACGGAGATGTGTCTCTGACCATTCAGGAGGTG : 300
Tim4eS CGGTACAACCTGCAGGGCTACATTTCCTACGGAGATGTGTCCCTGACCATTCAGGAGGTG : 300
Tim4L AAGGCAGAAGATGCGGGCACATACTGCTGCCGCGTGGAGATCCCAGGCTGGTTCAATGAC : 360
Tim4eS AAGGCAGAAGATGCGGGCACATACTGCTGCCGCGTGGAGATCCCAGGCTGGTTCAATGAC : 360
♦ hinge →
Tim4L ATCAAGAAGAACATTCAGCTGGCGGTGCTCGAAGTATTTGATGAGACAGCAAGTGGCACT : 420
Tim4eS ATCAAGAAGAACATTCAGCTGGCGGTGCTCGAAGTATTTGATGAGACAGCAAGTGGCACT : 420
Tim4L TATCTAGAAAAAATGGTGTCAACCAGTGCTCTCCAAGAATTTTCATCCAGTTTTCAAGCA : 480
Tim4eS TATCTAGAAAAAATGGTGTCAACCAGTGCTCTCCAAGAATTTTCATCCAGTTTTCAAGCA : 480
Tim4L GCTGATATGAGGACTGAAGGTGATGGCTTCTTCTACTCAACAGAGTTAATCCCTCTTCCT : 540
Tim4eS GCTGATATGAGGACTGAAGGTGATGGCTTCTTCTACTCAACAGAGTTAATCCCTCTTCCT : 540
Tim4L GAAGCAACAGCCCTGCCAGAGCCAACCACACTTCAGACACCGAAGTTAACCCTTGGTCCC : 600
Tim4eS GAAGCAACAGCCCTGCCAGAGCCAACCACACTTCAGACACCGAAGTTAACCCTTGGTCCC : 600
♦ IgV domain →
Tim4L TCAGCAATGAACACTGCATCAGAAACTGTTGTTCGAGGAGTGATAGGACAACCTGTCACA : 660
Tim4eS TCAG---------- : 604
Tim4L TTGCCTTGCTCCTACCAGGTGGCACAAGAGAAGGACATCTCCGATATGTGCTGGGGCAGA : 720
Tim4eS ---------- : 604
Tim4L GGCCCGTGCCCACACTCCAAGTGCAATGGCAAACTTTTGCACACCACTGGGAGCGAGGTG : 780
Tim4eS ---------- : 604
Tim4L ACATTCAGAACATCACAGCGGTACAACCTGCAGGGCTACATTTCCTACGGAGATGTGTCT : 840
Tim4eS ---------- : 604
Tim4L CTGACCATTCAGGAGGTGAAGGCAGAAGATGTGGGCACATACTGCTGCCGCGTGGAGATC : 900
Tim4eS ---------- : 604
♦ mucin domain →
Tim4L CCAGGCTGGTTCAATGACATCAAGAAGAACATTCAGCTGGTGGTGCTCGAAGCACCTCCA : 960
Tim4eS ---------- : 604
Tim4L TTGATGACAACAACCACGGGAAAAGCTCTCACTTCCCCCAACCATTTCAGAACAACGACT : 1020
Tim4eS ---------- : 604
Tim4L TTTGCTCTCCAAGAAACTTCTGATCTTCAAGCAACCACATGGACTGCTGTCCCCACAGTA : 1080
Tim4eS ---------- : 604
Tim4L GCACCTACAACCACTGGGTCTCCCCCAACGTTTGATGAGACAACAAGTGATAACGTTATG : 1140
Tim4eS ---------- : 604
Tim4L GAAGAAGTGATGACTATCAGTGCTCTCCCAGATTTTTCAACCAATTTCGAAACAAGTGAT : 1200
Tim4eS ---------- : 604
Tim4L GCATGGATTGAAGGTGAACCTGTGTTCTGCGCAACACAGCCCGAAGTGACTACTGAATTC : 1260
Tim4eS ---------- : 604

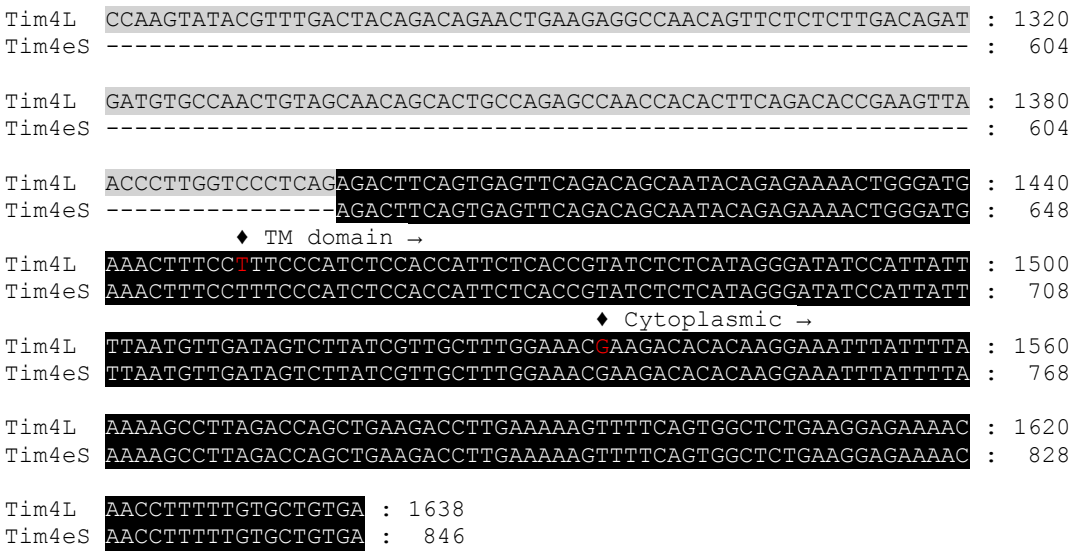


Figure 3.12. Alignment of Tim4L and Tim4eS. Both cDNAs were amplified from the same J line bird. Filled diamonds label the start-points of domains. The domains are labelled above the sequences. Identical residues are shown in black, less homologous residues in grey. Dashes show gaps.

3.3.9 Gene structure of chTim4 isoforms

The four chTim4 isoform cDNA sequences were compared with the chicken genome sequence in ENSEMBL to determine their gene structures. As shown in Figure 3.13, all four chTim4 isoforms are encoded by a single gene, which lies between nucleotides 11,536,376 and 11,551,073 on chromosome 13. The chTim4eL cDNA is derived from 12 exons with a long exon 3. ChTim4L is also encoded by 12 exons, but its exon 3 is 198 bp shorter than that of chTim4eL at the 5' end. ChTim4S is encoded by 9 exons, lacking exons 3, 4 and 5 of the chTim4L isoform. ChTim4eS is encoded by 8 exons with the same short exon 3 as chTim4L, but lacks exons 5, 6, 7 and 8 of the chTim4L isoform.

The exons encoding substructure of chTim4 proteins were also analysed, as showed in Figure 3.13. Exon 1 encodes the signal peptide, exons 2 and 5 respectively

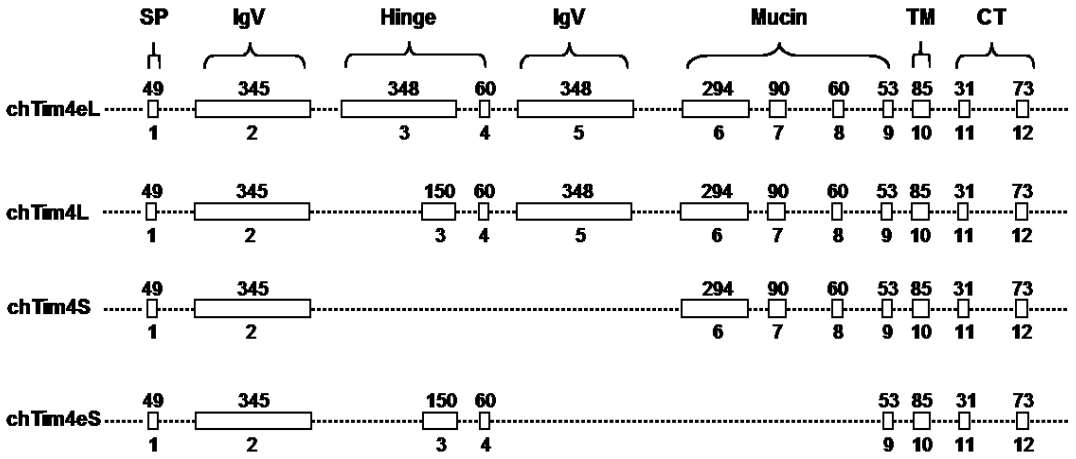


Figure 3.13. Exon usage of the four chTim4 isoforms. Boxes represent exons and are linked by introns, shown as dotted lines. The number above the box shows the length of each exon in base-pairs and that beneath the box indicates each exon. The sub-structures of the chTim4 proteins encoded by the exons are labelled on the top of exons, where SP = signal peptide, TM = transmembrane domain and CT = cytoplasmic tail.

the two repeated IgV domains, exons 3 and 4 the polypeptide hinge between the two IgV domains , exons 6-9 the mucin domain, exon 10 the transmembrane domain and exons 11 and 12 the cytoplasmic tail. Therefore, both chTim4eL and chTim4L proteins have two repeated IgV domains, but the former has a longer hinge than the latter. By contrast, both chTim4S and chTimeS only have one IgV domain; the former has a longer mucin domain, identical to those of chTim4eL and chTim4L, whereas the latter has a very short mucin domain, but maintains the same hinge as chTim4L.

3.3.10 Expression of recombinant chTim family proteins

Recombinant proteins were expressed in the supernatants of COS-7 cells transiently transfected with relevant plasmids, using DEAE/dextran as described in

section 2.7.1. The presence of recombinant protein in the COS-7 cell supernatants was tested by ELISA as described in section 2.6.2.

The chTim1 extracellular domain Ig fusion protein was expressed at higher levels than those of the chTim4S extracellular domain Ig fusion protein, even though all transfection procedures were as similar as possible. However, the expression levels of the chTim4L-2IgV and chTim4L-extracellular domains were very low or not detectable in the capture ELISA assay (Figure 3.14). When comparing yields of the different subunit chTim molecules, as shown in Figure 3.15, the recombinant mucin domain protein had the highest expression levels. The IgV domain protein had similar levels of expression to those of the extracellular domain proteins. The variation in levels of fusion protein expression may be a consequence of PS binding sites in the IgV domain, by means of which the fusion protein could partially bind to PS on the membrane of the intracellular cell apparatus or the inner layer of the cell membrane during expression, eventually impacting on secretion levels of the protein in the supernatants.

To determine if the chTim4L-Ig fusion protein does not express at all, the plasmids were electroporated into COS-7 cells, as described in section 2.7.2. The transfected cells were then fixed and probed with an anti-human IgG antibody and anti-PDI (which stains the ER apparatus) or anti-GM130 (for Golgi apparatus) antibodies, followed by fluorescently labelled secondary antibodies, as described in section 2.8. The stained cells were analysed by confocal microscopy. The protein was present in ER but not in the Golgi apparatus (Figure 3.16).

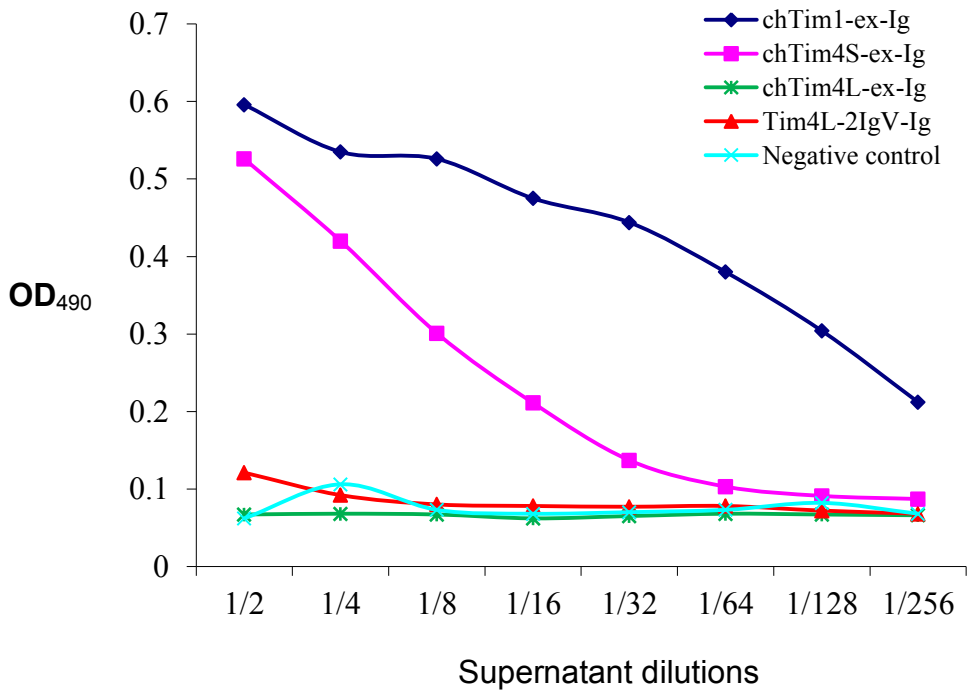


Figure 3.14. Detection of expression of chTim family proteins by a capture ELISA. The Ig fusion proteins in transfected COS-7 supernatants, containing extracellular domains of chTim1 (blue line), chTim4S (pink line), chTim4L (red line) or the IgV domains of chTim4L (2IgVs, light blue line), were captured on ELISA plates, pre-coated with unlabelled goat anti-human IgG antibody. The bound fusion proteins were then probed with an HRP-conjugated goat anti-human IgG antibody. The HRP activities were detected by the substrate OPD and read at OD 490 nm. Negative control (green line) is untransfected COS-7 supernatant.

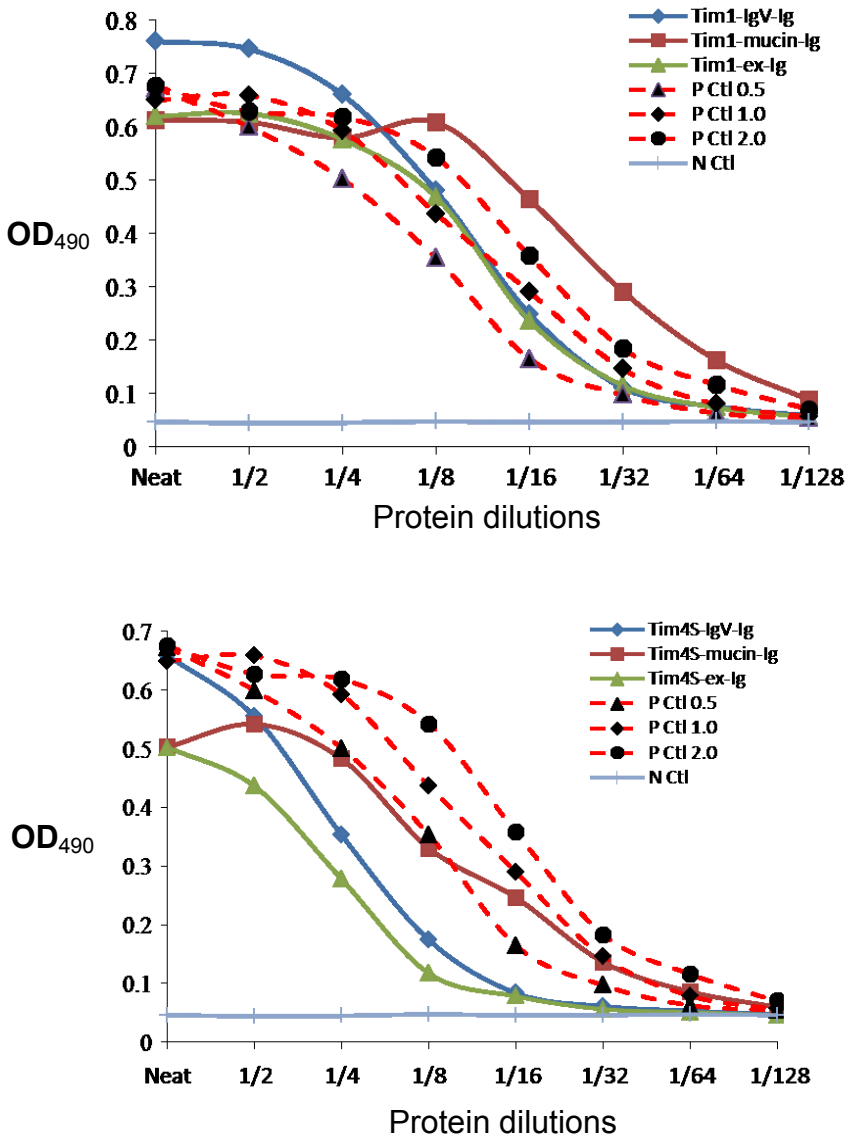


Figure 3.15. COS-7 cell transient expression of recombinant chTim domain proteins analysed by capture ELISA. The top graph shows Ig-fused Tim1 domains, with Ig-fused Tim4S domains in the bottom graph. The IgV domains are shown as blue lines, the mucin domains as solid red lines, the extracellular domains as green lines. A purified Ig-fused chicken CD86 protein at concentration of 0.5 (triangle), 1.0 (diamond) and 2.0 (dot) µg/ml was used as positive control (P Ctl, red dashed lines). Negative control (N Ctl, light blue lines) was supernatant from untransfected COS-7 cells.

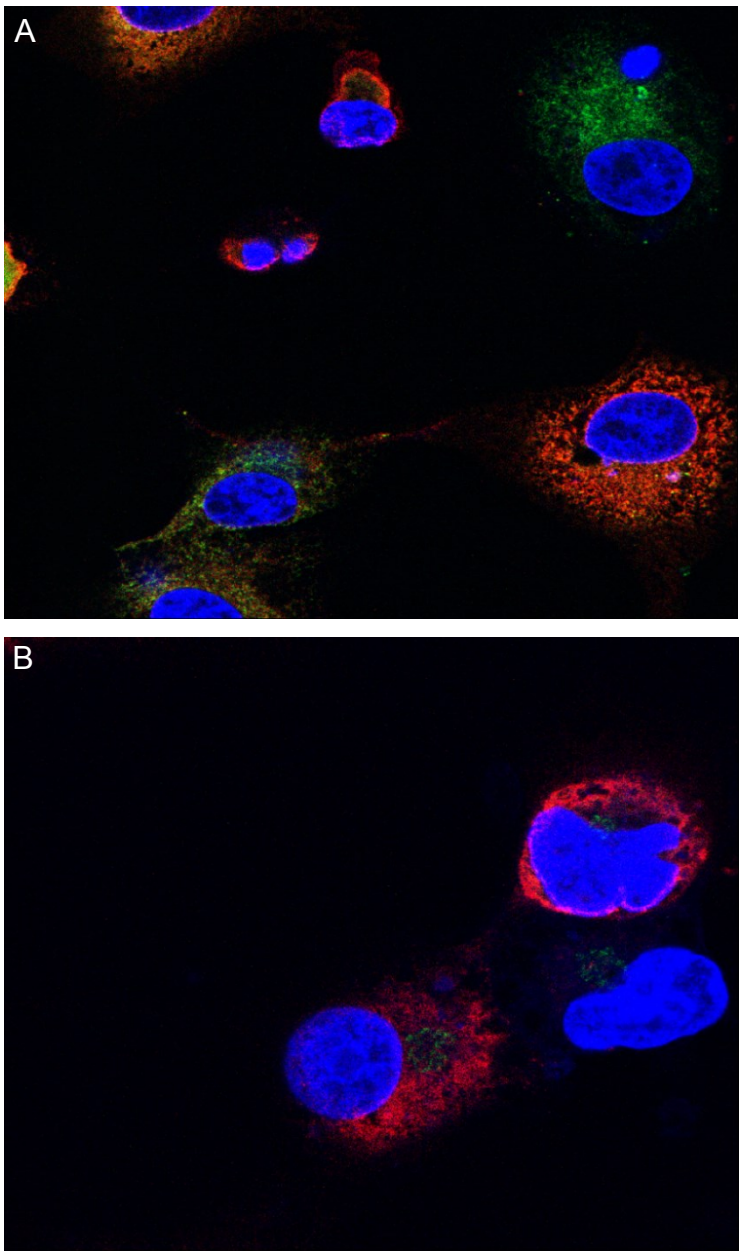


Figure 3.16. Confocal microscopy of co-localisation of chTim4L-extracellular-Ig fusion protein in COS-7 cells. Transfected cells were co-stained with a biotinylated goat anti-human IgG Ab, followed by an Alexa-fluor 568-conjugated streptavidin in red, and either (A) a mouse anti-PDI (for ER apparatus) or (B) a mouse anti-GM130 (for Golgi apparatus), followed by detection using an Alexa fluor 488 conjugated goat anti-mouse Ab in green. Nuclei were stained with DAPI in blue. Therefore, negatively-transfected cells are in green and blue; positively-transfected cells are in orange and blue (A) or in red, green and blue (B).

3.3.11 Large-scale protein expression from stably transfected CHO cells

Three Ig-tagged fusion plasmids, including the chTim1-, chTim4S-extracellular and the chTim4L hinge domains, and two His6-tagged plasmids containing extracellular domain of either chTim1 or chTim4S were transfected into CHO-K1 cells to establish stable transfectants (for details see Table 3.3). Single colonies were screened by capture ELISA, as described in section 2.6.2, for Ig-fusion protein-producing colonies.

In each transfection, 960 colonies (ten 96-well plates) were screened. 48 colonies with the highest ELISA optimal density (OD) readings were then selected and grown up in 24-well plates. After further screening, 12 colonies were chosen and grown up in 6-well plates. After a third round of screening, 6 colonies were chosen to grow up in T75 tissue culture flasks and one colony, finally, which consistently scored highly in all rounds of screening, was chosen to express recombinant protein at a large scale by miniPERM culture (section 3.2.4). For the His6-tagged fusion proteins, in each transfection, ten 96-well plates were seeded for growing up single colonies. After counting the colony numbers in each well under the microscope, the supernatants from the wells, with only one colony per well, were chosen and dotted into nitrocellulose membranes. Dot-blotting assays were performed as described in section 2.6.3. Figures 3.17 (A, B) and 3.18 (A, B) show the selection of colonies secreting His6-tagged proteins by dot-blot. 17 chTim1-His6- and 5 chTim4S-His6-expressing colonies were chosen to grow up in 24-well plates. The supernatants were further tested by slot-blotting, as shown in Figures 3.17 (C) and 3.18 (C). Stably transfected colonies 1 (expressing chTim1) and 5 (expressing chTim4S) were selected to culture in the miniPERM bioreactor for large-scale protein expression.

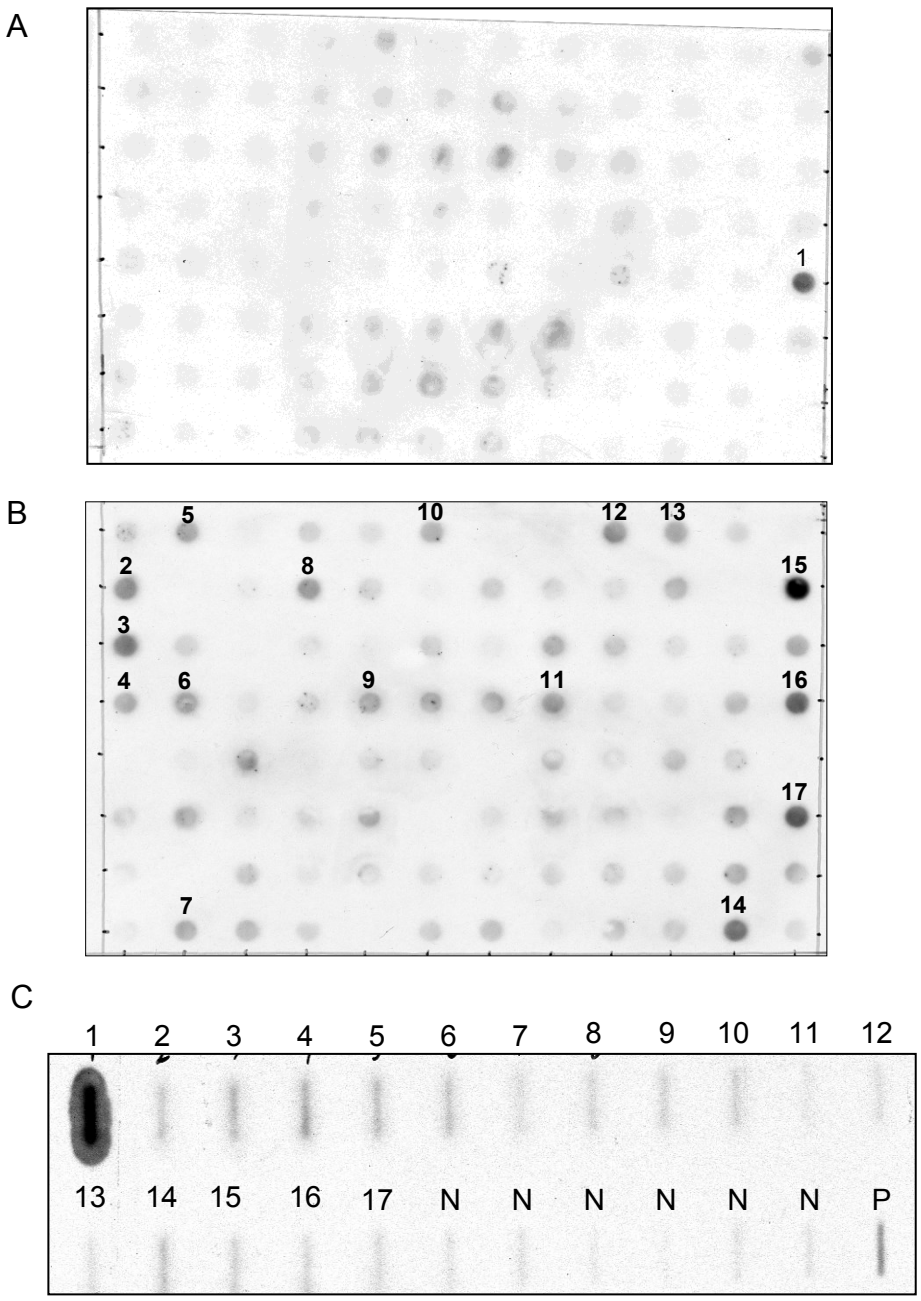


Figure 3.17. Selection of stably transfected CHO colonies expressing high levels of chTim1-His6 protein by dot- (A, B) or slot- (C) blotting. (A) and (B) show results of screening single colonies grown on 96-well plates. (C) shows a further screen of the colonies grown on 24-well plates. The numbers above the slots and dots correspond to the same colonies. Negative controls (N) were Ham's F12 medium, and the positive control (P) was 500 ng of purified His6-tagged protein (chCD86-His6).

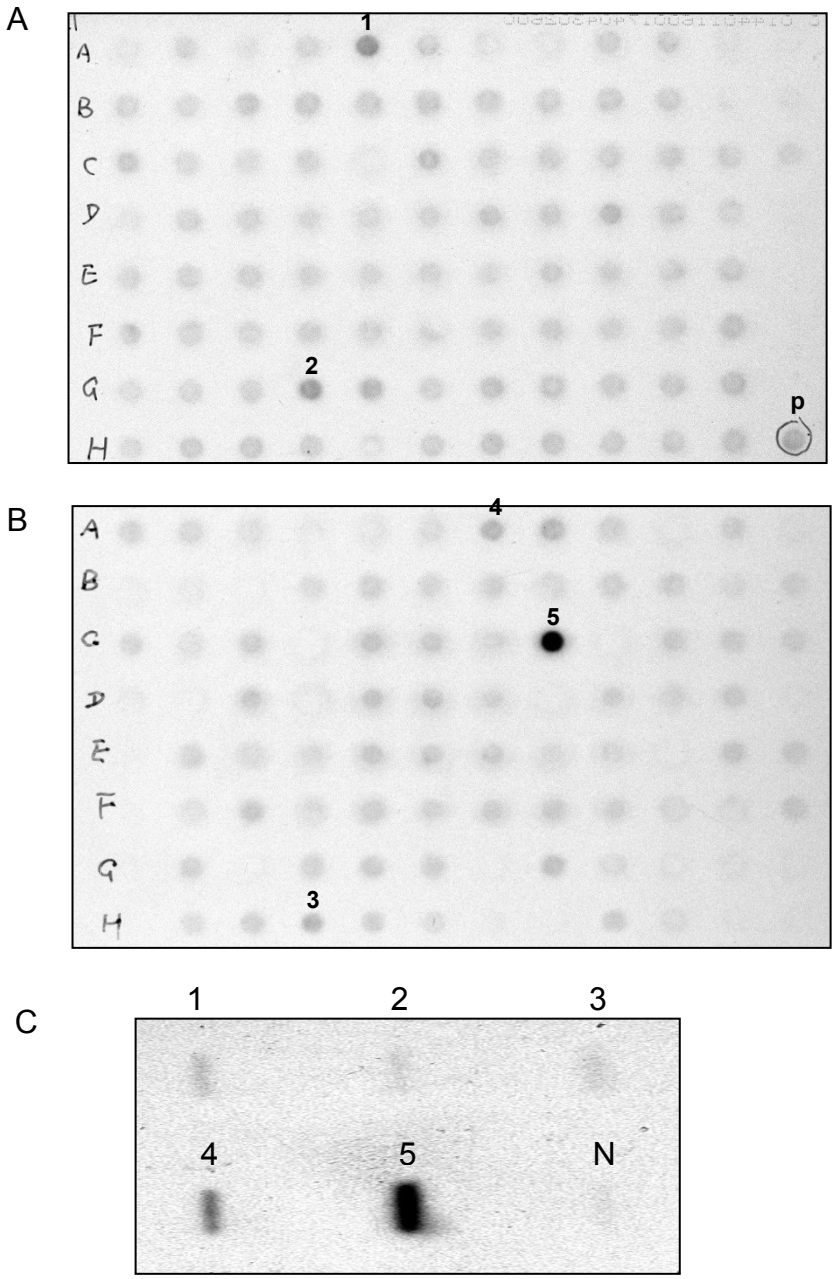


Figure 3.18. Selection of stably transfected CHO colonies expressing high levels of chTim4S-His6 protein by dot- (A, B) or slot- (C) blotting. (A) and (B) show results of screening single colonies grown on 96-well plates. (C) shows a further screen of the colonies grown on 24-well plates. The numbers above the slots and dots correspond to the same colonies. Negative control (N) was Ham's F12 medium, and the positive control (P) was 500 ng of purified His6-tagged protein (chCD86-His6).

The supernatants from the miniPERM culture were further tested by slot-blotting, as shown in Figure 3.19.

3.3.12 Characterisation of purified recombinant chTim fusion proteins

Recombinant proteins from the miniPERM supernatants were purified on a protein G column for the Ig-tagged proteins (section 2.9) or by Ni-NTA slurry for the His6-tagged proteins (section 3.2.6). The purified proteins were then electrophoresed on an SDS-PAGE gel, followed by Coomassie blue staining (section 2.6.5) to monitor protein purity or western blot (section 2.6.6) to estimate the approximate molecular weight. The 231 aa of the monomerised IgG1 Fc tag have a calculated molecular mass of 26.4 kDa and two N-linked glycosylation sites, resulting in the protein migrating as 30-35 kDa in an SDS-PAGE gel. This Ig-tag was fused to the chTim1- or chTim4S-extracellular domains or the chTim4L-hinge domain, with molecular masses of 46.5, 56.4 and 34.6 kDa, respectively, predicted with Protparam software (http://web.expasy.org/protparam).

As a result of O-linked or N-linked glycosylation (or both), the Ig-tagged chTim1- and chTim4S-extracellular domain fusion proteins migrated at approximately 72 kDa and 95 kDa respectively under reducing or non-reducing conditions (Figures 3.20A, 3.21A and B). The chTim4L-hinge-Ig protein migrated at 55 kDa under reducing conditions (Figures 3.20B and 3.21C). His6-tagged chTim1- and chTim4S-extracellular domain fusion proteins migrated at approximately 46 and 66 kDa respectively (Figures 3.22A and B). To avoid bovine Ig contamination, Ig-depleted Foetal bovine serum (FBS) was used in the cell cultures instead of normal FBS. This modification resulted in a high purity of the Ig-tagged proteins after

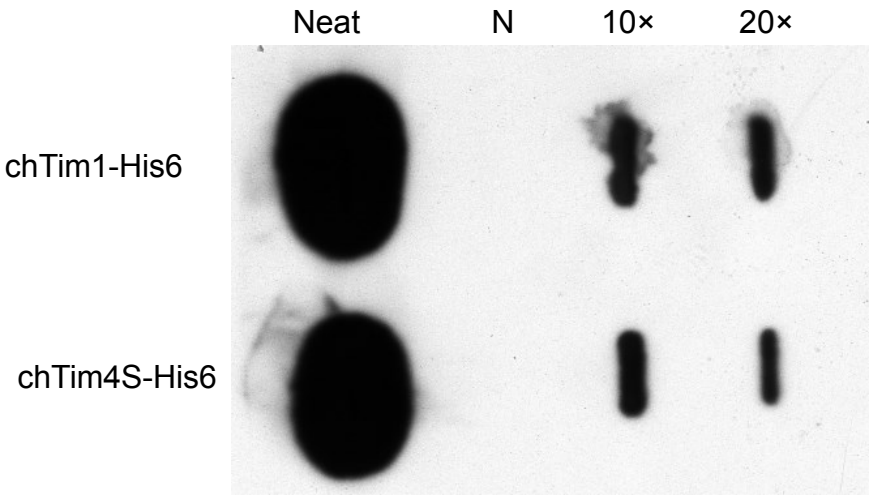


Figure 3.19. Expression of chTim-His6 fusion proteins with miniPERM cultures. Stable CHO transfectants were cultured with miniPERM bioreactors for massive protein expression, followed by analysis of protein in their supernatants with slot-blotting. The diluted or undiluted supernatant was dotted onto a nitrocellulose membrane and probed with a Penta-his antibody. Neat = undiluted, 10× = ten times dilution, 20× = twenty times dilution, N = negative control (supernatant from untransfected CHO cells).

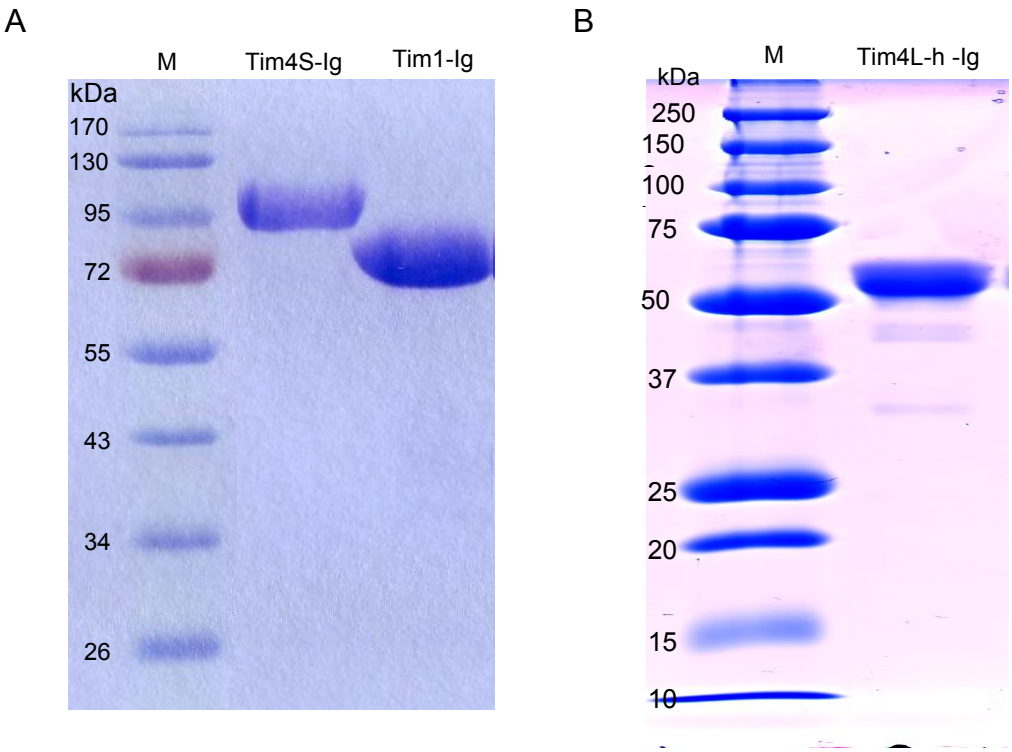


Figure 3.20. Coomassie blue staining of purified chTim-Ig fusion proteins. The chTim-Ig fusion proteins were expressed by stably transfected CHO cells cultured in a miniPERM bioreactor. The purified proteins were resolved on 4-12% SDS-PAGE gels under reducing conditions. (A). ChTim1- and chTim4S-extracellular domain Ig fusion proteins, M = Pageruler prestained protein ladder (Fermentas). (B). ChTim4-hinge domain Ig fusion protein. M = Precision Plus protein standard (all blue, BioRad).

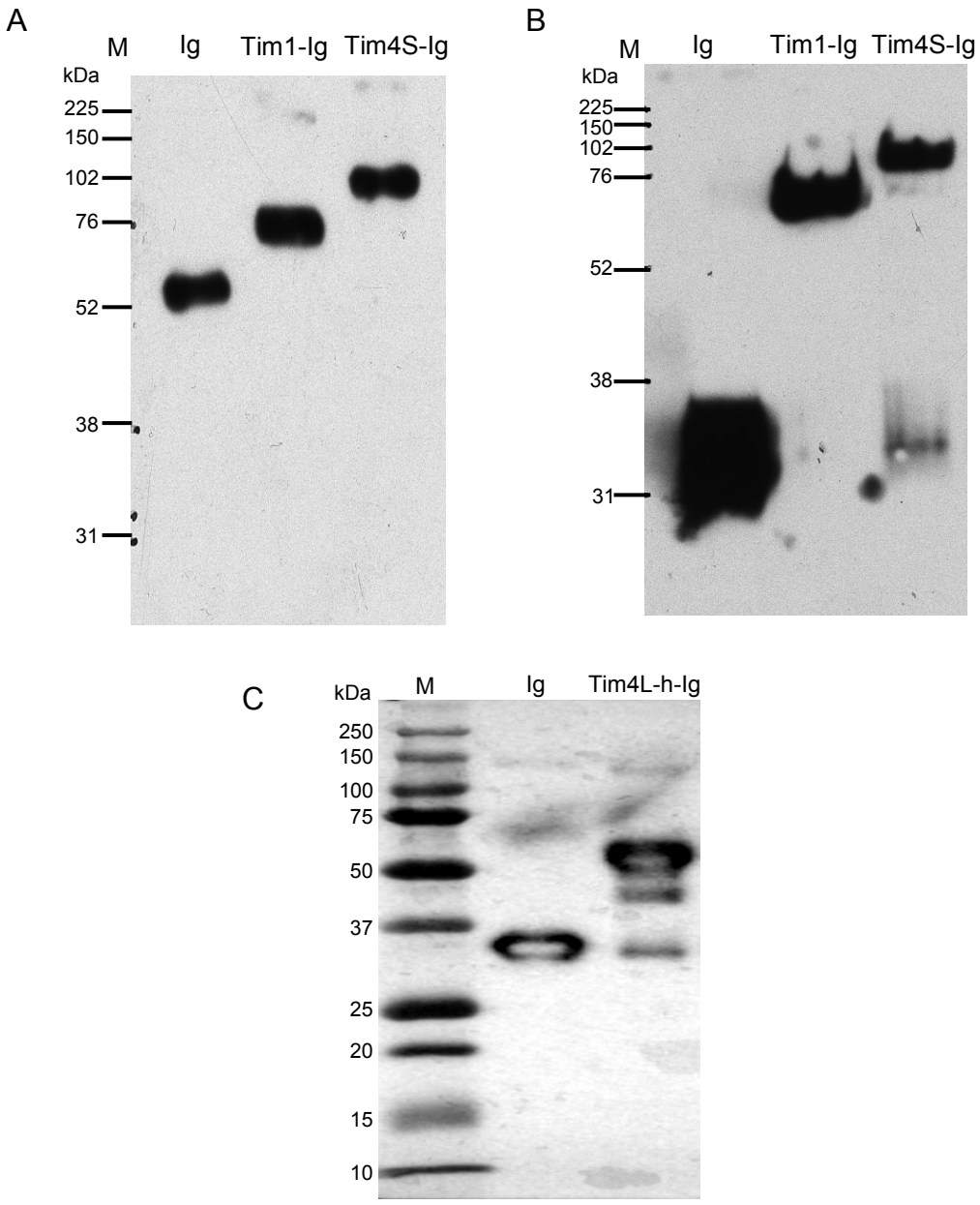


Figure 3.21. Western blot analysis of purified chTim-Ig fusion proteins. Purified chTim1- and chTim4S-extracelluar domain Ig fusion proteins were resolved on SDS-PAGE (A) under non-reducing conditions, or (B) under reducing conditions; (C) chTim4L-hinge-Ig protein resolved under reducing conditions. Proteins were then probed by HRP-conjugated goat anti-human IgG antibody, followed by ECL detection. Ig = human IgG1 (Pro100-Lys330) (R&D Systems). M = Full-range rainbow protein ladder (A, B) (Invitrogen) or Precision Plus protein standard (all blue) (C). Chemiluminescence was exposed on X-ray films (A, B) or directly detected by a G:Box Chemi (Syngene).

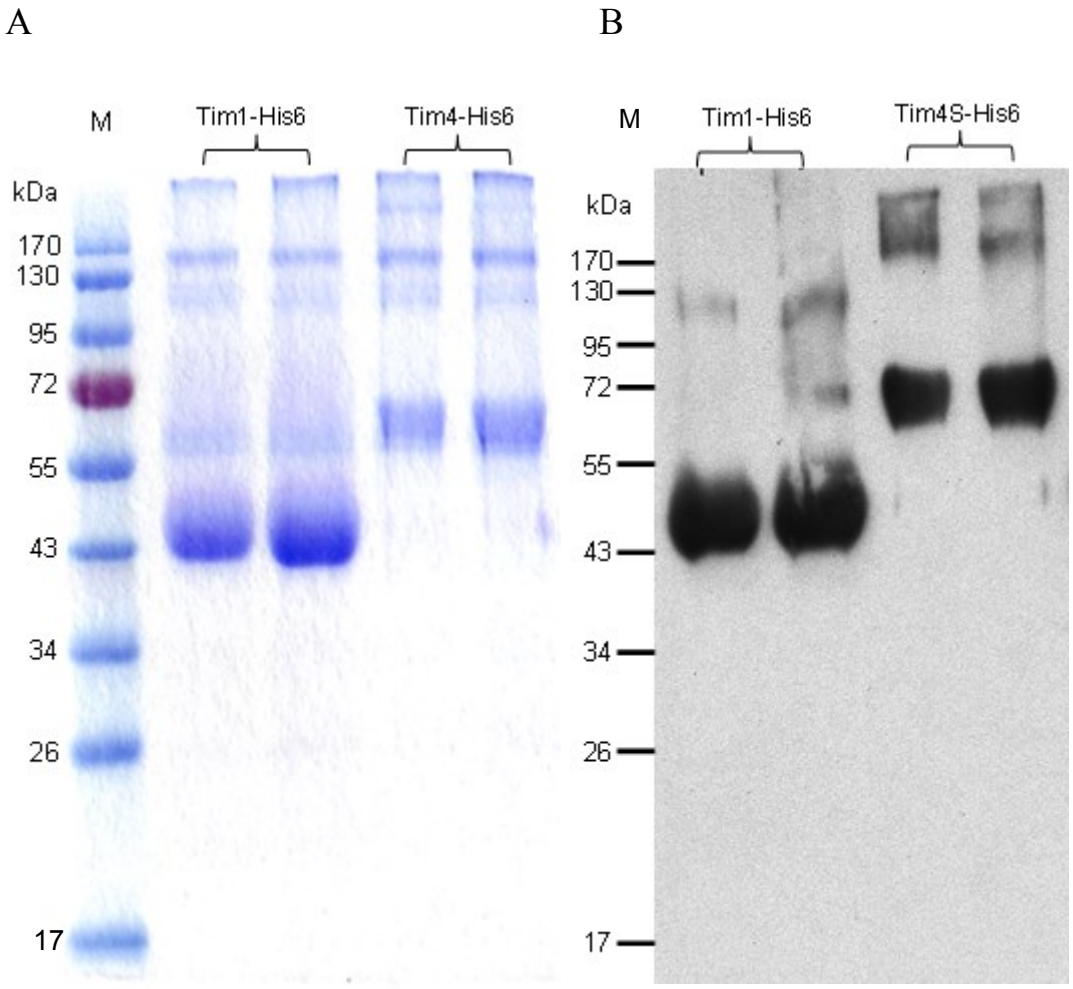


Figure 3.22. Purified chTim1- and chTim4S-extracellular domain His6 fusion proteins. The Ni-NTA affinity-purified chTim-His6 fusion proteins were electrophoresed on 4-12% SDS-PAGE gels under reducing conditions, followed by (A) Coomassie blue staining or (B) western blotting. A Penta-his antibody was used to probe the chTim-His6 proteins in western blot, followed by HRP-conjugated goat anti-mouse IgG antibody. ECL was used to detect HRP activity. M = Pageruler prestained protein ladder (Fermentas).

purification on a protein G column (Figure 3.20A and B). Affinity purified His6-tagged proteins using Ni–NTA resin were less pure (Figure 3.22A).

3.4 Discussion

As described previously, the murine Tim family lies on chromosome 11, which is homologous to the human chromosomal region 5q23-35, which contains the human Tim family genes. Both regions are linked to atopy and asthma (Loots et al., 2000; Yokouchi et al., 2000; Walley et al., 2001). The human 5q region contains a large number of cytokine genes, including IL-9, IL-12 p40 and the Th2 cytokine cluster, which contains the genes that encode IL-4, -5 and -13, as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (Loots et al., 2000; Kelly et al., 2000). Chromosome 13 is the syntenic chicken chromosome encoding IL-9, IL-12 p40, the Th2 cytokine cluster (IL-4, -5 and -13), GM-CSF and IL-3 (Kaiser et al., 2005; Avery et al., 2004; Balu et al., 2003; 2011). The chicken Tim locus maps to a syntenically conserved region on chromosome 13.

The number of Tim family members is different in human and mouse species. Orthology of Tim genes between them has been studied by phylogenetic analysis of IgV domain. Murine Tim4-8 genes together were evolved independently; murine Tim3, a homology of human Tim3, is also an ancient Tim gene lineage. However, murine Tim1 and Tim4, homologous to human Tim1 and Tim4 respectively, are closely related and cluster in a different branch, suggesting they may be a consequence of paralogous duplication to each other in early evolutionary stage in both species. Later on, a paralogous duplication of murine Tim1 resulted in a novel murine Tim2 (Shakhov et al., 2004; Kuchroo et al., 2003). However, a phylogenetic

tree of full-length Tims (Figure 3.2B) showed that Tim1 and Tim3 in both human and mouse are more closely related to each other and equally related to human and mouse Tim4. This cluster pattern of Tim molecules is consistent with evolution of their biological functions. Because the role of mucin domains in Tims is to help IgV domains properly interacting with their ligands, therefore, they co-evolved; Tim1 and Tim3 have functional similarities with expressing on the surface of $CD4^+$ T cells and regulating immune response of these cells, consistently to their evolutionary relationship. Chicken evolutionarily lost functional Tim3, but has Tim1 and Tim4, which are homologous to their counterparts in human and mouse.

Polymorphisms are present in mammalian Tim molecules and are associated with susceptibility to disease. The mucin domain in Tim1 is longer in BALB/c mice, which are more susceptible to Th2-driven airway hypersensitivity, than in DBA/2 and C57BL/6 mice, which develop less airway reactivity following antigen challenge (McIntire et al., 2001). Like mice, an insertion of six aa forming a long human Tim1 mucin domain (157insMTTTVP) resulted in protection against asthma and allergy in subjects previously exposed to HAV (McIntire et al., 2003). Unlike Tim1, murine Tim3 has two splice variants, a full-length Tim3 (encoding a membrane protein) and a short soluble Tim3 (encoding a soluble Tim3), lacking the mucin and transmembrane domains of full-length Tim3. This soluble Tim3 binds with high affinity and avidity to its receptors and serves to inhibit effector Th1 cell responses, which are crucial for the induction of peripheral tolerance (Sabatos et al., 2003). Chicken Tim1 insertion/deletion variants have yet to be identified. However, chicken Tim4 was originally identified in these studies as two novel splice variants, a short and a long isoform; the latter has an extra IgV domain when compared to the former.

They are both encoded by the same gene (Figure 3.13). ChTim4L is encoded by 12 exons. Among them, exons 2 and 5 are highly homologous, encoding two IgV domains of chTim4L protein. They are linked by polypeptides encoded by exon 3 and 4 (Figure 3.13). However, chTim4S is encoded by 9 exons, lacking exons 3, 4 and 5 of the chTim4L.

Comparison of the putative aa sequences of chTim4S and chTim4L indicated that they have identical sequences in their signal peptide, mucin and cytoplasmic domains. However, a single aa difference is present in the transmembrane domain and there are more differences in the IgV domains; the IgV domain of chTim4S is not entirely identical to either IgV domain of chTim4L (Figures 3.6 and 3.8B). To determine if aa variations between chTim4S and chTim4L are also present in different genetic backgrounds, chTim4S and chTim4L cDNAs were amplified again from an outbred J line bird. Similarly to the cDNA clones from the line 7_2 bird, aa variations, this time in the mucin and IgV domains were also present between chTim4S and chTim4L from the J line bird (Figure 3.8A). Several aa residues were persistently variable between the IgV domain of chTim4S and either of the IgV domains of chTim4L in both lines (Figure 3.8B). A possible explanation for these differences is that chTim4S and chTim4L are splice variants of the same gene but that they are transcribed from different parent alleles, which are heterozygous at the chTim4 locus.

It is notable that exons 4 and 8 of chTim4L and the intronic sequences around them are highly homologous (Figure 3.10). As mentioned previously, the elements associated with regulation of RNA splicing are normally located close to an exon to be spliced at the 5′ and 3′ ends of introns, such as the branch site and splice sites.

Therefore, the sequence similarity implies that exons 4 and 8 may have equal chance of being spliced during RNA splicing, probably resulting in the different alternative splice variants to chTim4S and chTim4L. An attempt was made to clone this predicted chTim4 splice variant by RT-PCR. Eventually, a shorter version of chTim4 cDNA was cloned from splenic RNA from a J line bird (Figure 3.11). This new chTim4 isoform was even shorter than chTim4S and thus named chTim4eS. It was encoded by 8 exons. As predicted, the chTim4eS mRNA excludes exons 5, 6, 7 and 8 of chTim4L. There is only a single nucleotide polymorphism between exons 4 and 8 (Figure 3.10). The chTim4eS mRNA includes exon 4 but excluded exon 8, as it has the same nucleotide residue as that in exon 4.

An extra longer isoform of chTim4 (chTim4eL) was also identified with a longer exon 3 than that of chTim4L but almost identical to chTim4L in the remainder of the molecule. It was not detected when chTim4S and chTim4L were first amplified, probably due to low abundance in the RNA sample. Adherent splenocytes stimulated with rchCD40L seem to express high levels of chTim4eL (Figure 3.13A), although it is yet to be determined if the high expression levels of chTim4eL were a consequence of rchCD40L stimulation or if chTim4eL-expressing cells were enriched in adherent splenocytes. ChTim4 has 12 constitutive exons and a longer constitutive exon 3 is present in chTim4eL, whereas the other chTim4 variants have a shorter alternatively splicing exon 3. RT-PCR results indicated that chTim4eL is not abundantly expressed in normal spleen tissue (Figure 3.7A), although it contains all of the constitutive exons; chTim4S and chTim4L are the most abundant forms present in spleen tissue and rchCD40L-stimulated suspension splenocytes respectively (Figure 3.7A). There may be regulatory elements in exon 3, such as an

exonic splicing enhancer (ESE)/silencer (ESS), or in the intronic region flanking exon 3, such as an intronic splicing enhancer (ISE)/silencer (ISS), that may control splicing to give a shorter form of exon 3.

HAV infection studies indicated that both murine Tim1-IgV and -mucin domains are involved in virus invasion, but play different roles. A fusion protein containing only the IgV domain could block the binding of HAV to cells, but a similar fusion protein containing only the mucin domain did not. However, a fusion protein consisting of both IgV and mucin domains of Tim1 blocked ten-fold more infection than the IgV domain alone and efficiently triggered viral uncoating, a necessary event for viral entry, suggesting the IgV domain of Tim1 has a binding site for HAV and the mucin domain may enhance this interaction (Silberstein et al., 2001; 2003). In addition, mucosal addression cell adhesion molecule (MAdCAM)-1, which has a similar structure to that of the Tim family, has two distinct ligands, one for its Ig domains and one for its mucin domain, and each ligand has a separate function (Berg et al., 1993; Shaw and Brenner, 1995). As described before (Chapter 1), each mammalian Tim molecule has multiple ligands and their binding sites are mapped to their IgV domains; Tim1 ligands include PS, IgA, LMIR5, Tim4 and Tim1 itself as well as HAV virus; Tim2 ligands include H-ferritin and Sema4A; Tim3 ligands include PS and galectin-9 and Tim4 ligands include PS and LMIR5. Through interaction with multiple ligands, these Tim proteins play complex roles (Freeman et al., 2010; Kane, 2010). To determine if different domains of the chicken Tim family proteins play differential roles, fusion proteins containing different chTim domains were expressed. Chicken Tim molecules have potential multiple N- and O- linked glycosylation sites, suggesting they may be highly glycosylated

proteins. Therefore, mammalian expression systems were used to express these recombinant chTim fusion proteins to allow post-translation modifications and hopefully generation of functional proteins. The resulting chTim fusion proteins will be used to investigate their biological and immunological roles; for example, do they act as PS receptors to mediate dead cell clearance and as costimulatory molecules to stimulate immune cell proliferation in Chapter 4. The chTim fusion proteins will also be used to generate anti-chTim mAbs to investigate chTim molecule expression on chicken immune cells in Chapter 5.

However, recombinant chTim4L fusion proteins, containing IgV (two IgVs) and entire extracellular domains, proved difficult to detect in the supernatants of transfected mammalian cells (Figure 3.14), although they were expressed by these cells (Figure 3.16). A possible explanation is that the double PS-binding sites in the chTim4L protein may result in the protein binding tightly to PS, either on the membrane of the ER apparatus to prevent intracellular transportation of the fusion protein, or on the inner layer of the transfected cell membrane to limit the ability of soluble protein to be secreted into the supernatant. The unique hinge between the two IgV domains of chTim4L was fused to an Ig tag for protein expression and used to generate monoclonal antibodies in Chapter 5.

Chapter 4. Characterisation of the chicken Tim family molecules.

4.1 Introduction

The first member of the Tim family molecules to be discovered was Tim1, originally identified as a receptor of hepatitis A virus (HAV) in African green monkey and named HAVCR-1 (Kaplan et al., 1996). It has a structure similar to that of mucosal addressin cell adhesion molecule 1 (MAdCAM-1). Its extracellular region consists of an IgV domain and a mucin domain (Ichimura et al., 1998). The IgV domain has an HAV binding site, while the mucin domain enhances binding, which results in 10 times more HAV neutralisation from the entire extracellular domain than from the IgV domain alone (Silberstein et al., 2001; 2003). Murine Tim1 is expressed on activated but not naïve $CD4^+$ T cells, especially on differentiated Th2 cells. Th1 and Th17 cells express little or no Tim1 (McIntire et al., 2001; Umetsu et al., 2005; Meyer et al., 2005a; Nakae et al., 2007b). Other cell types, such as mast cells, B cells and tubular epithelials cells post-kidney injury, also express variable levels of Tim1. Tim1 mRNA can be detected in invariant natural killer T cells (Khademi et al., 2004). Tim1, as a costimulatory molecule, plays a role in regulation of Th2 immune responses and cytokine production (Umetsu et al., 2005). *In vivo* administration of an agonist Tim1 mAb together with antigen also enhances antigen-specific T cell responses (Umetsu et al., 2005). Interestingly, Tim1 mAbs also show profoundly different effects on airway inflammation. A murine Tim1 mAb which recognises an epitope in the mucin domain can exacerbate airway

inflammation in a mouse model of asthma, but a murine Tim1 mAb mapping to the IgV domain can block inflammation in the same model (Sizing et al., 2007).

In mammals, Tim4 is a natural ligand of Tim1. It is preferentially expressed on antigen presenting cells and plays a role in costimulation of activated T cells to proliferate through Tim1-Tim4 interaction (Meyer et al., 2005a). The crystal structure of the Tim4 IgV domain revealed a metal ion-dependent ligand binding site where phosphatidylserine (PS) binds (Santiago et al., 2007a). PS exposure is a marker for apoptotic cells. As a receptor for PS, Tim4 mediates the phagocytosis of apoptotic cells by macrophages or DCs to maintain the body's homeostasis (Kobayashi et al., 2007). Tim4 also plays an important role *in vivo* as a mediator of tolerance. *In vivo* administration of Tim4 mAb, post-dexamethasone-induced apoptosis in the murine thymus, blocks binding of PS and results in the development of autoantibodies (Miyanishi et al., 2007).

In this Chapter, the expression patterns of the chicken Tim mRNAs in various tissues and cell types will be studied using quantitative real-time RT-PCR (qRT-PCR) to address if they are immune-related molecules and which cells express which molecules. The chTim fusion proteins generated and described in Chapter 3 will also be used to investigate chTim ligands and to study their potential roles in the phagocytosis of dead cells and costimulatory activity, as described for their mammalian counterparts.

4.2 Materials and Methods

4.2.1 Measurement of chTim expression on tissue panel by qRT-PCR

Primers and probes for qRT-PCR (Table 4.1) were designed using PrimerExpress software (Applied Biosystems). All probes were labelled with the fluorescent report dye 5-carboxyfluorescein (FAM) at the 5' end and the quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. The primers were obtained from Sigma and the probes from Eurogentec. One-step Taqman assays were performed using the Reverse Transcriptase qPCR Master Mix kit (Eurogentec) according to the manufacturer's instructions, except that 10 µl reactions were used. The specific products were amplified and detected using the ABI PRISM7700 Sequence Detection System (Applied Biosystems) with the following thermal cycling profile: 48°C for 30 s, 95°C for 20 s, and then 40 cycles of 95°C for 3 s followed by 60°C for 30 s. Tissues were taken from a 6-week-old line 7_2 bird and total mRNA prepared with RNeasy mini kits (QIAGEN) including on-column digestion with DNase (QIAGEN). Each assay was performed in triplicate. The results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance threshold (ΔRn). For variation caused by sampling and RNA preparation, the Ct value of 28S rRNA product from each individual RNA sample assay was used to standardise the Ct value of the target gene in the same RNA sample. To normalise the RNA levels between samples within an experiment, the mean Ct value of 28S product was calculated by pooling values from all samples in the same experiment. For standard curves, RNA isolated from HD11 cells stimulated by LPS, or COS-7 cells transfected with the cloned full-length cDNA in pCI-neo, was used as template for amplification. The threshold level was

RNA target	Probe/primer sequence (5'-3')	Exon boundary
28S	Probe (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA) Forward primer GGCGAAGCCAGAGGAAACT Reverse primer GACGACCGATTTGCACGTC	
chTim1	Probe (FAM)-TGGCTTACAGGCCCCACAGTGTCG-(TAMRA) Forward primer TCCTGGACTGGATCCTTCTGA Reverse primer TCTGACCAACCTCTCCCTTCA	1/2/3
chTim4S	Probe (FAM)-ACTGTAGCAACAGCACTGCCAGAGCCA-(TAMRA) Forward primer AGTTCTCTCTTGGCAGATGATGTG Reverse primer AAGGGTTAACTTCGGTGTCTGAAG	7/8
chTim4L	Probe (FAM)-CCTGCCAGAGCCAACCACACTTCA-(TAMRA) Forward primer TCCCTCTTCCTGAAGCAACAG Reverse primer GCTGAGGGACCAAGGGTTAAC	3/4/5

Table 4.1. Primers and probes for qRT-PCR.

set to the same value for each plate where a given gene was assayed. The following equation was used to calculate the adjusted Ct value (Nt): Nt = Ct + (S/S') (Nt'-Ct'), where Nt is the adjusted value, S and S' are the slopes of the standard curve semilog plots of Ct against loge (initial amount of RNA) for the chTim RNAs and 28S RNA, and Ct and Ct' are respectively the Ct measurement for the chTim RNAs and 28S RNA in the samples.

4.2.2 Binding of Tim protein to PS detected by dot-blot

L-α-phosphatidylcholine (PC) from egg yolk, 3-sn-phosphatidylethanolamine (PE) from bovine brain, L-α-phosphatidylinositol (PI) ammonium salt solution from bovine liver and 3-sn-phosphatidyl-L-serine (PS) sodium salt from bovine brain were

purchased from Sigma-Aldrich and dissolved in chloroform. Phospholipid binding assays on a nitrocellulose membrane were performed as previously described (Miyanishi et al., 2007). Each phospholipid solution (2 μg) was dotted onto the membrane strips, with PBS as a blank control, and air-dried at room temperature. After blocking the membrane with 1% casein/PBA for 1 h, the membranes were incubated for 1 h with COS-7 cell supernatants containing 1 μg/ml of chTim1-Ig or chTim4S-Ig fusion protein. The membranes were washed with PBST. Binding proteins were detected with HRP-conjugated goat anti-human IgG Ab (Southern Biotech) and developed with an ECL kit (GE Healthcare) as described in section 2.6.3.

4.2.3 Binding of Tim protein to PS detected by ELISA

A solid-phase ELISA assay to test interactions between chTim proteins and phospholipids was carried out as previously described (Miyanishi et al., 2007). Each phospholipid stock solution was diluted in ethanol to a concentration of 2 μg/ml, and added to each well of a 96-well ELISA plate, 50 μl per well, and air-dried. The plate was blocked with 3% BSA/PBS (w/v) at 4°C overnight. The purified chTim1- or chTim4-Ig fusion protein was two-fold serially diluted in 1% BSA/PBS on the lipid-coated plate. Each dilution was performed in triplicate wells, and incubated for 1 h at room temperature. The plate was washed with PBST, the bound protein was detected by adding 1 μg/ml of goat anti-human IgG Ab conjugated to HRP and peroxidase activity was detected with the substrate OPD, as described in section 2.6.1.

4.2.4 Phagocytosis assay

Full-length chTim1 cDNAs, cloned in expression vector pcIneo, were transfected into NIH-3T3 cells using the Gene Pulser Xcell electrophoration system (Bio-Rad) with the pre-set programme for 3T3 cells, as described in section 2.7.2. Transfected cells, or untransfected cells as a control, were seeded at 1×10^3 cells per well on a 4-well chamber slide (Sigma-Aldrich) and cultured for 48 h. Apoptotic cells were prepared by culturing chicken splenocytes with 50 μg/ml ConA for 6 h, and then mixing with with 4',6-diamidino-2-phenylindole (DAPI) solution (300 nM in PBS) for 5 min to label cell nuclei, following which labelled cells were washed twice with PBS. Apoptotic cells, 1×10^6 cells per well, were added to 3T3 cells in the chamber slides and incubated for 30 min at 37ºC, 5% CO_2. To remove non-phagocytosed cells, the 3T3 cell layers were vigorously washed with PBS five times, and cells phagocytosed by the 3T3 cells were visualized with an ultraviolet microscope (Leica).

4.2.5 Labelling the Ig fusion protein with fluorescent dye

A fluorescent dye, Alexa fluor 647, was conjugated to recombinant Ig proteins using a Zenon Human IgG Labeling Kit (Invitrogen), according to the manufacturer's instructions. Purified Ig protein solution (1 μg) was mixed with 5 μl labelling reagent, incubated for 5 min, and the reaction terminated by adding 5 μl blocking reagent for 5 min. The complex was then ready for staining cells for flow cytometric analysis.

4.2.6 PS inhibition of chTim4S protein binding to stimulated splenocytes

Chicken splenocytes were isolated as described in section 2.3.5.1 and cultured in complete RPMI1640 medium supplemented with or without 3 μg/ml rchCD40L protein or 3 μg/ml ConA (Sigma-Aldrich) for 48 h as described in section 2.3.5.4. PS was diluted in PBS and sonicated in an ultrasonic liquid processor (Misonix) at full power for 5 min to make up a 1.2 mg/ml (1.5 M) solution. The PS solution was then mixed with chTim4S-Ig at a ratio of 150 nmoles (0.12 μg) PS per μg protein in PBS containing 10% FCS, 2.5 mM $CaCl_2$ for 1 h at room temperature to block the PS binding site. A chCTLA4-Ig fusion protein was included as a PS-blocking control or an irrelevant Ig protein (human IgG Fc, R&D Systems) as a negative control. The blocked and unblocked recombinant proteins were then adjusted to 10 μg/ml with PBS containing 10% FCS, 2.5 mM $CaCl_2$. These protein solutions were used to immunostain cultured splenocytes for flow cytometric analysis as described in section 2.10. A FTIC-conjugated goat anti-human IgG antibody (SouthernBiotech) was used to detect the bound protein on cells.

4.2.7 Splenocyte costimulation assay

Anti-chicken CD3 and CD28 antibodies (SouthernBiotech) were immobilized on a flat-bottomed 96-well tissue culture plate as described previously (Meyers et al., 2005a). Briefly, CD3 and/or CD28 antibodies were diluted to 1.0 μg/ml in sterile PBS. The antibody solution, or PBS as controls, 50 μl per well, was added onto the plate and incubated for 3 h at 37ºC; the plate was then vigorously washed three times with sterile PBS to remove any unbound antibodies.

Chicken splenocytes were isolated as described in section 2.3.5.1 and adjusted to a density of 2.0×10^6 cells/ml. The cell suspension (100 µl/well) was added to the antibody-coated plate with or without the addition of chTim4S-Ig fusion protein in different concentrations, with an irrelevant Ig protein (chBAFFR-Ig, a gift from Dr John Young, IAH, Compton, UK) as control. The cells were cultured at 41ºC, 5% CO_2. At 48 h of culture, the cells were pulsed with 1 µCi of [^{3}H] thymidine per well for 16 h. Incorporation of [^{3}H] thymidine [corrected counts per minute (c.c.p.m.)] was measured in a β-scintillation counter (Perkin Elmer).

4.2.8 Sorting chicken splenocyte subsets with MACs cell sorter

Spleens were taken from 6-week-old 7_2 line birds for isolation of splenocytes (section 2.3.5.1). The cells were sorted into different subsets using an autoMACS Separator (Miltenyi Biotec). As per manufacturer's instructions, cells were labelled with magnetic microbeads (Miltenyi Biotec) prior to cell sorting. Briefly, for each magnetic labelling, 2×10^7 cells were resuspended in 200 µl 1% BSA/PBS and 1 µg antibody added (mouse anti-chicken cell surface marker, SouthernBiotech), mixed thoroughly and incubated on ice for 20 min. The cells were washed three times with 1 ml of PBS, pelleted and resuspended in 160 µl of running buffer (PBS containing 0.5% BSA (w/v), 2 mM EDTA, 0.09% azide). Magnetic microbeads (40 µl), coupled with goat anti-mouse IgG antibody, were then added to the cell suspension and mixed thoroughly. The cells were incubated on ice for 15 min to allow the beads to bind to the cells. After washing three times as before to remove unbound beads, the cells were then resuspended in 500 µl running buffer and transferred to a 15 ml Falcon tube for cell sorting on an autoMACS Separator with a positive separation

programme. The sorted cells were then pelleted again for total RNA preparation, as described in section 2.4.

4.2.9 rchCD40L-stimulated adherent and suspension splenocytes

Splenocytes were isolated a 6-week-old line7_2 bird, as described in section 2..3.5.1, and incubated in complete RPMI 1640 medium at 37ºC, 5% CO_2 for 3 h, as described in section 2.3.5.4. Resulted suspension cells were then transferred to new tissue culture flasks and cultured with or without the addition of 2 µg/ml rchCD40L for 72 h at 41ºC, 5% CO_2; the adherent cells were also cultured in complete RPMI 1640 medium (section 2.3.5.4) with or without the addition of 2 µg/ml rchCD40L for 72 h at 41ºC, 5% CO_2.

4.3 Results

4.3.1 Tissue mRNA expression patterns of chTim1 and chTim4

The mRNA expression levels of chTim1 and chTim4 were measured in various tissue RNA samples by one-step qRT-PCR, as described in section 4.2.1. As shown in Figure 4.1A, chTim1 mRNA was present in high abundance in all the immune and non-immune organs tested, except for muscle. To detect chTim4, two sets of primers and probes were designed: one set, across exons 7 and 8, should detect all the chTim4 isoforms except for chTim4eS; another set, across exons 3, 4 and 5, should detect chTim4eL, chTim4L and chTim4eS. As shown in Figure 4.1B, analysis with the first set of qRT-PCR reagent indicated that chTim4 is abundantly expressed in all immune and non-immune tissues tested, except for muscle. In contrast, the second set of reagents detected expression of chTim4 mRNA only in the

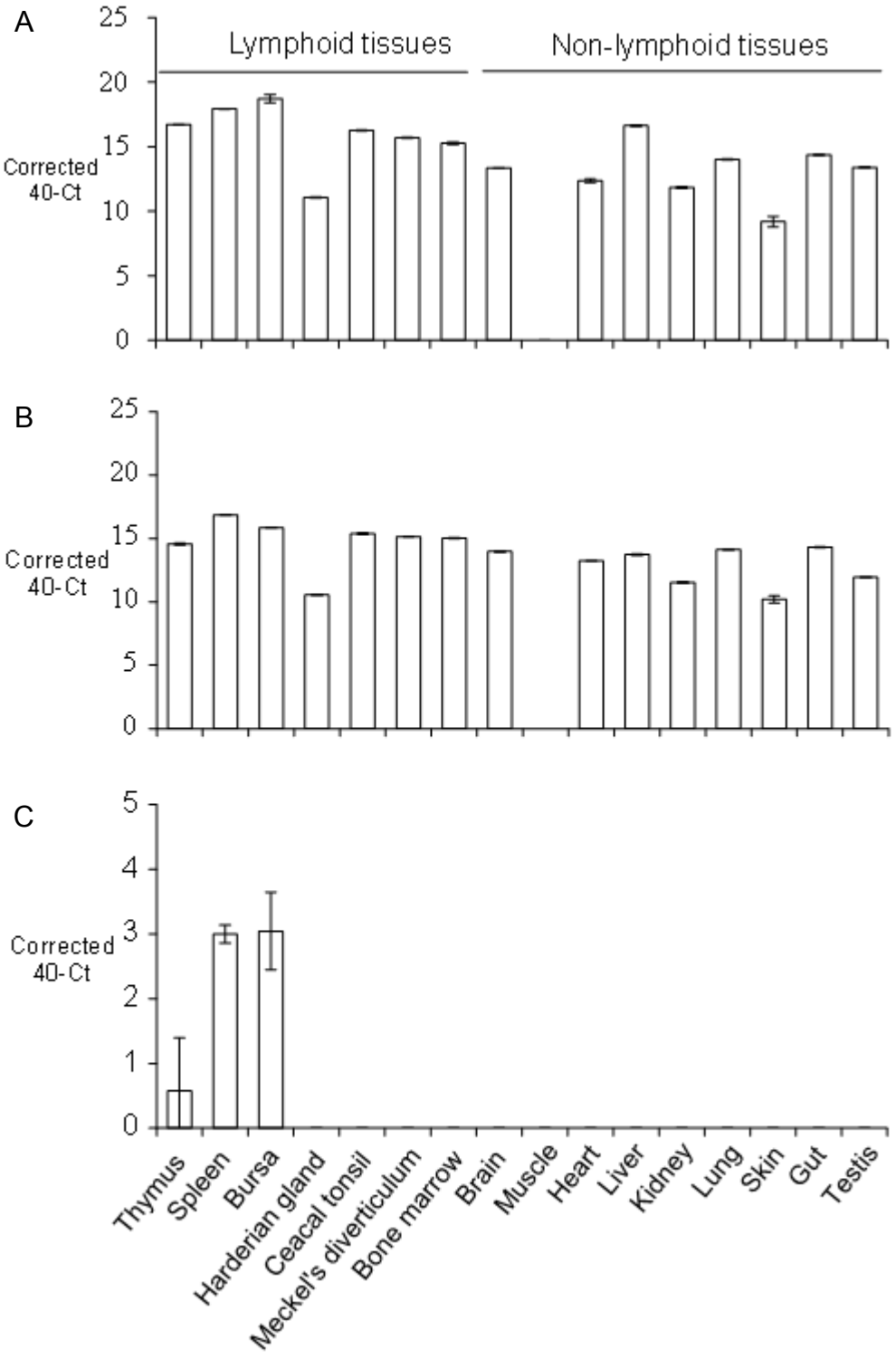


Figure 4.1. Quantification of chTim mRNA in various tissues, as measured by real-time RT-PCR, with results expressed as corrected 40-Ct. (A) chTim1, (B) chTim4S and (C) chTim4L. Error bar indicates the standard error of three repeats of each sample.

spleen, bursa of Fabricius and the thymus at low levels (Figure 4.1C), suggesting the chTim4 isoforms containing exons 3 and 4 (chTim4eL, chTim4L and chTim4eS) are either not expressed or expressed at levels below the sensitivity of the assay in most tissues. By contrast, qRT-PCR across exons 7 and 8 detected high levels of chTim4 expression in these tissues, suggesting abundant chTim4S expression in these tissues.

4.3.2 mRNA expression patterns of chTim1 and chTim4 in immune cells

qRT-PCR analysis of various chicken tissues revealed chTim mRNAs were highly expressed in the spleen (Figure 4.1). To determine which cell subsets in the spleen express chicken Tim mRNAs, a set of RNAs (kind gifts from Dr Zhiguang Wu) prepared from $CD4^+$, $CD8^+$, $TCR1^+$, $TCR2^+$, $TCR3^+$ and $Bu\text{-}1^+$ cells, isolated by positive selection from the spleens of 6-week-old inbred line 7_2 chickens, were examined by qRT-PCR. A set of RNAs from APCs (a kind gift from Dr Zhiguang Wu) was also analysed for chTim expression, including bone marrow-derived DCs, peripheral blood-derived macrophages, LPS- or rchCD40L-stimulated DCs or macrophages (for 24 h) prepared as described before (Wu et al., 2010a). In splenic subsets, as shown in Figure 4.2, $TCR1^+$ and $Bu\text{-}1^+$ cells express chTim mRNAs at the highest levels, with low levels of expression in $CD4^+$ and $CD8^+$ cells and no detectable expression in $TCR2^+$ and $TCR3^+$ cells. APCs also express all chTims, but there was no detectable expression of those chTim4 isoforms containing exons 3 and 4 (chTim4eL, chTim4L and chTim4eS) in peripheral blood derived-macrophages under any conditions (Figure 4.2C).

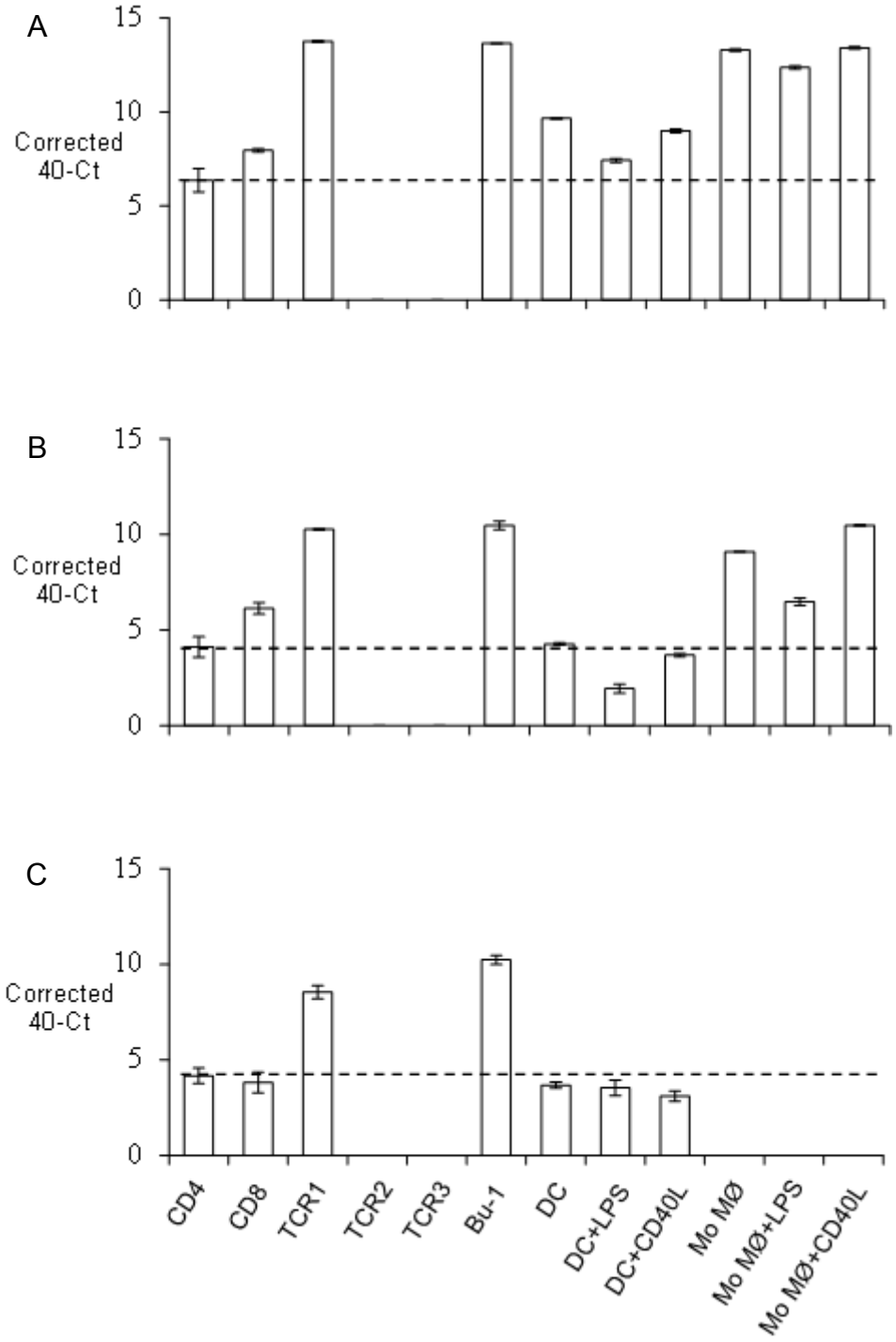


Figure 4.2. Quantification of chTim mRNA in various immune cell subsets, as measured by qRT-PCR, with results expressed as corrected 40-Ct. (A) chTim1, (B) chTim4S and (C) chTim4L. Dashed lines show the chTim expression levels in $CD4^+$ T cells for comparison. Error bar indicates the standard error of three repeats of each sample.

To confirm the qRT-PCR findings and to attempt to distinguish expression of the different isoforms, chTim4 mRNA expression was reanalysed by RT-PCR using a new panel of RNAs, which were prepared from various splenocyte subsets from 6-week-old line 7_2 birds, as described in section 4.2.8, including $CD3^+$, $CD4^+$, $CD8\alpha^+$, $CD8\beta^+$, $TCR1^+$, $TCR2^+$, Bu-1^+ and $KUL01^+$ cells. RNAs from rchCD40L-stimulated adherent and suspension splenocytes (section 4.2.9) were also included to see if stimulation varied expression of chTim4 isoforms. All RNAs, 1μg per sample, were then reversely transcribed into cDNAs (section 2.5.1) which were then used as templates to amplify full-length chTim4 cDNAs with the primers Tim4-F1/R1, as described in section 3.2.1. As shown in Figure 4.3, T cells ($CD3^+$) and T cell subsets, including $CD8\alpha^+$, $CD8\beta^+$ and $TCR1^+$, preferentially transcribe chTim4S mRNA. Similarly, Bu-1^+ and $KUL01^+$ cells also preferentially express chTim4S mRNA. However, $CD4^+$ and $TCR2^+$ cells express low or undetectable levels of chTim4 mRNA. These results are consistent with the previous qRT-PCR analysis of the cell panel (Figure 4.2), and suggest that chTim4S is the constitutive isoform expressed by immune cells. By contrast, all cultured adherent and suspension splenocytes preferentially expressed chTim4L mRNA (Figure 4.3).

4.3.3 Distribution of chicken Tim ligands on splenocytes

To investigate the contribution of different parts of the chicken Tim molecule in binding to potential receptors, recombinant fusion proteins, containing only the IgV domain, only the mucin domain or the entire extracellular domain of chTim1 or chTim4S, with a human IgG Fc tag, were expressed from transiently transfected COS-7 cells. These Ig fusion proteins were used to stain chicken splenocytes. As

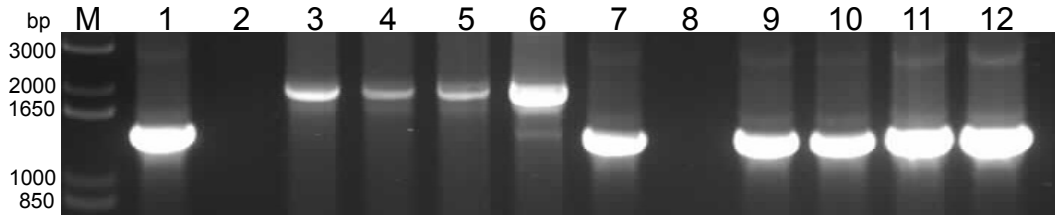


Figure 4.3. RT-PCR analysis of chTim4 expression in various splenic cell types. Total RNA was extracted from sorted splenocyte subsets, as well as stimulated or unstimulated adherent and suspension splenocytes. Full length chTim4 cDNAs were amplified by RT-PCR with the primers Tim4-F1/R1. 1 = $TCR1^+$, 2 = $TCR2^+$, 3 = rchCD40L-stimulated adherent cells, 4 = non-stimulated adherent cells, 5 = non-stimulated suspension cells, 6 = rchCD40L-stimulated suspension cells, 7 = $CD3^+$, 8 = $CD4^+$, 9 = $CD8\alpha^+$, 10 = $CD8\beta^+$, 11 = $Bu\text{-}1^+$, 12 = $KUL01^+$. M = TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen).

shown in Figure 4.4A, flow cytometric analysis indicated that the Tim1-IgV and -mucin fusion proteins have no detectable staining on splenocytes, while the chTim1-extracellular-Ig fusion protein had a slight, but increased binding when compared to controls (human IgG Fc, R&D Systems). This suggests that the splenic cell population expressing the chTim1 receptor is small in number. Similarly, the chTim4S-mucin domain fusion protein did not bind to splenocytes (Figure 4.4B). By contrast, the chTim4S-IgV-Ig fusion protein positively stained a population of splenocytes. A larger population was stained by the entire Tim4S-extracellular-Ig fusion protein (Figure 4.4B). These binding patterns are consistent with those of mammalian Tim4, where the IgV domain possesses the ligand binding site and high affinity binding requires both the IgV and mucin domains (Silberstein et al., 2001; 2003; Meyers et al., 2005a; Santiago et al., 2007a; 2007b).

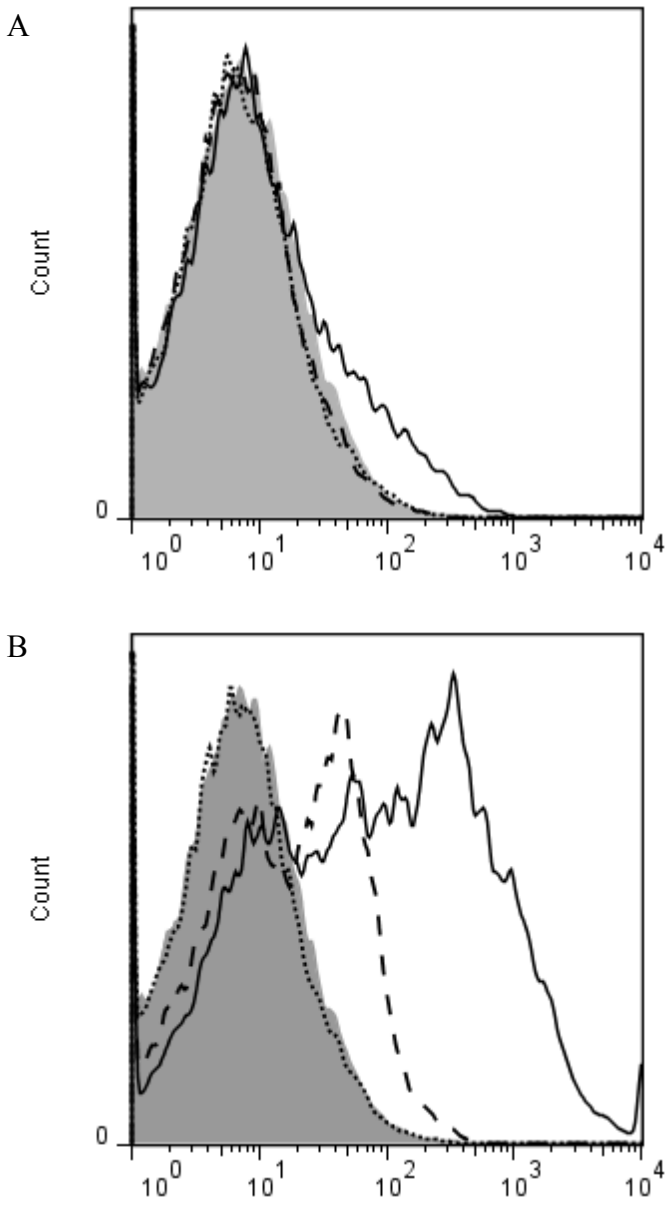


Figure 4.4. Splenocytes were stained with recombinant fusion proteins of chTim1 or chTim4S. (A) Splenocytes were stained with human IgG1 Fc (filled histogram), chTim1-IgV domain (dashed line), chTim1-mucin domain (dotted line) and chTim1 entire extracellular domain (solid line). (B) Splenocytes were stained with human IgG Fc (filled histogram), chTim4S-IgV domain (dashed line), chTim4S-mucin domain (dotted line) or chTim4S entire extracellular domain (solid line). The detecting Ab was a goat anti-human IgG-FITC. The flow cytometric results here represent one experiment of three repeats.

4.3.4 Upregulation of chTim4S ligand expression on stimulated splenocytes

Ligands of the chTim molecules, presumably chTim1, chTim4 and/or PS, are expressed on splenocytes, as shown by flow cytometric analysis using the recombinant chTim fusion proteins (Figure 4.4). To address if these ligands are regulatively or constitutively expressed on splenocytes, splenocytes were stimulated with rchCD40L for B cell, or ConA for T cell, proliferation. The expression levels of the chTim4S ligand(s) were measured by flow cytometric analysis, immunostaining the cells with recombinant chTim4S-Ig fusion protein and anti-chicken Bu-1 or CD3 antibodies. As shown in Figure 4.5A, stimulation with rchCD40L resulted in a higher frequency of Bu-1^+ splenocytes when compared with non-stimulated splenocytes, suggesting rchCD40L stimulation successfully enhanced B cell activation and proliferation.

The frequency of chTim4S-Ig^+ Bu-1^+ cells was also increased when compared with non-stimulated splenocytes, suggesting rchCD40L-stimulated B cells upregulated expression levels of the chTim4S ligand(s). Similarly to rchCD40L stimulation, ConA stimulation also resulted in an increased frequency of $CD3^+$ chTim4S-Ig^+ cells when compared with non-stimulated splenocytes, as shown in Figure 4.5B, suggesting ConA stimulation could also induce upregulated expression levels of the chTim4S ligand(s) on activated T cells.

4.3.5 PS is a ligand of the chTim proteins, as analysed by dot-blot and ELISA

Multiple sequence alignments (Figure 3.4 and 3.5) indicated that the

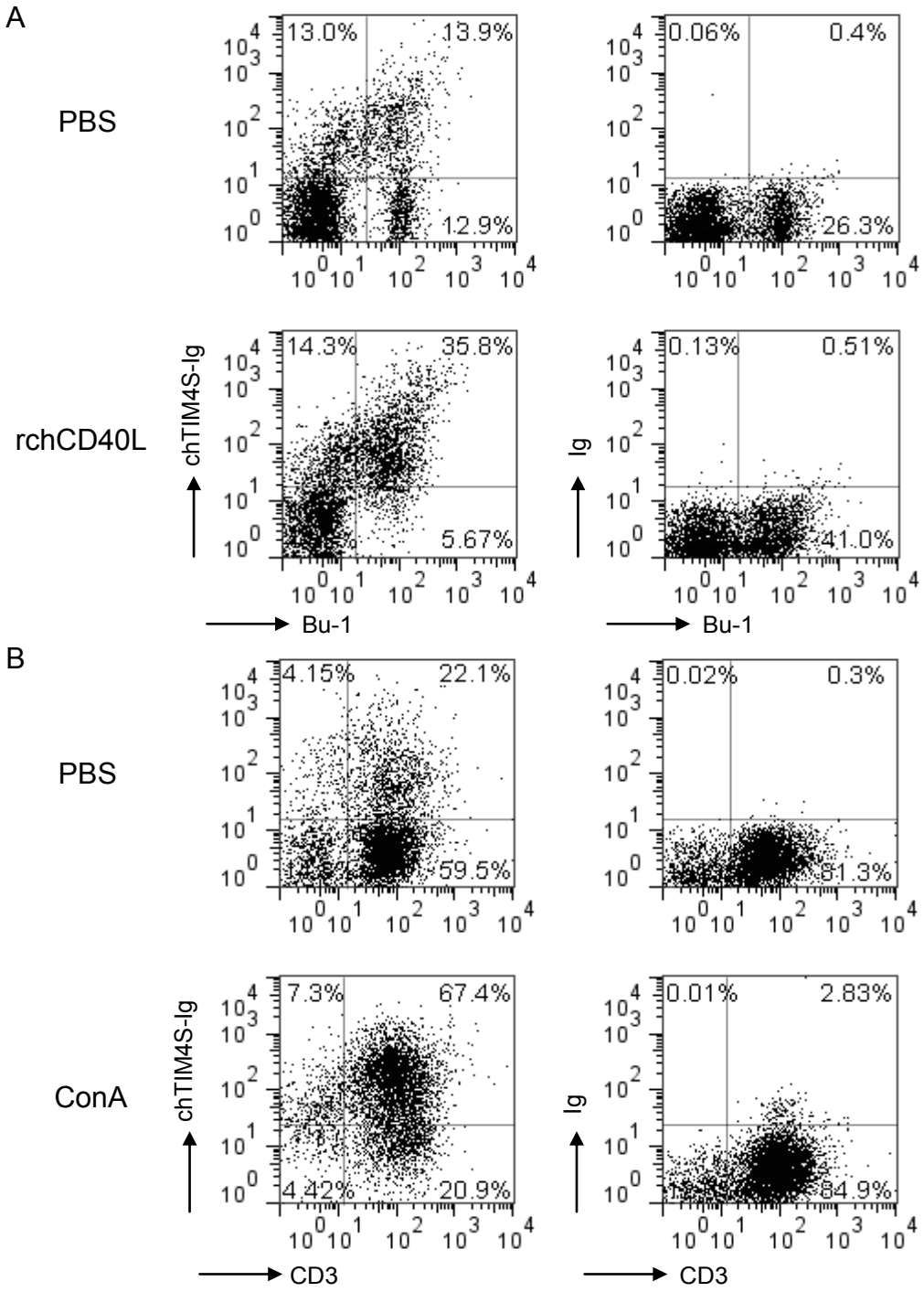


Figure 4.5. The ligands of Tim4S were upregulated after stimulation, as measured by flow cytometry. Splenocytes were cultured with or without (A) rchCD40L or (B) ConA for 48 h. The cells were stained with Tim4S-Ig fusion protein, or human IgG1 Fc as negative control, conjugated to Alexa fluor 647, and either anti-Bu-1-FITC (A) or anti-CD3-FITC (B) mAbs. The flow cytometric results here represent one experiment of four repeats.

predicted aa sequences of chTim1 and chTim4S contain a conserved PS-binding site (Santiago et al., 2007a; 2007b; Kobayashi et al., 2007). To test whether this predicted site in the chicken is functional, the interaction of chTim1 or chTim4S with PS was examined by a protein-phospholipid overlay assay as described in section 4.2.2. Various phospholipids were dotted on nitrocellulose membranes and the chTim fusion proteins were used to probe them. As shown in Figure 4.6, the chTim1-Ig and chTim4-Ig fusion proteins specifically bind to PS but not to the other phospholipids, except for slight binding to PE by chTim4S.

To confirm if PS is a ligand of chTim4S and chTim1, solid-phase ELISA was performed on phospholipid-coated plates as described in section 4.2.3. As the results show in Figure 4.7, the chTim1- and chTim4-Ig fusion proteins displayed dose-dependent binding to PS with only background levels to the other phospholipids tested.

4.3.6 ChTim4S-Ig recognises PS exposed on the surface of apoptotic chicken cells

The protein-phospholipid overlay studies demonstrated that chTim1 and chTim4 specifically bind to PS and not to other phospholipids (Figures 4.6-4.7). Stimulation of chicken splenocytes also revealed that ConA and rchCD40L could upregulate the expression levels of chTim4S ligands on the cell surface (Figure 4.5). It seems a reasonable hypothesis that cell death is inevitable during culture, leading to PS exposure on the cell surface. To investigate this, splenocytes were cultured with a high dose of ConA (50 μg/ml) for 6 h to mimic activation-induced cell death (Himer et al., 2010). The stimulated cells were then stained with PI to detect dead

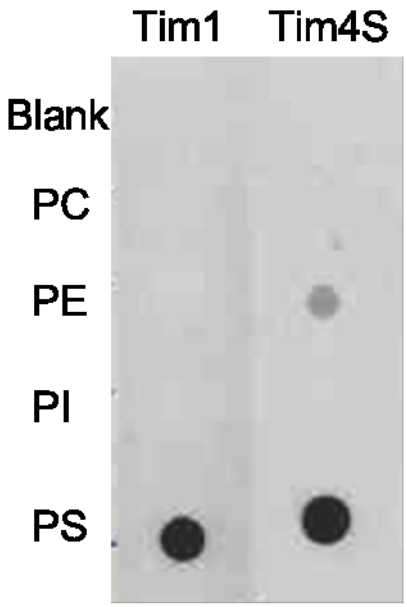


Figure 4.6. Dot-blot analysis of interaction of chTim1-Ig and chTim4S-Ig fusion proteins with phospholipids. Each phospholipid was dotted onto nitrocellulose membrane strips, with PBS as negative control. COS-7 cell supernatants containing either chTim1-Ig or chTim4S-Ig fusion proteins were incubated with the membranes. Binding of fusion protein was detected with an HRP-conjugated goat anti-human IgG antibody. The HRP activity was detected with an ECL kit. PC = Phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine.

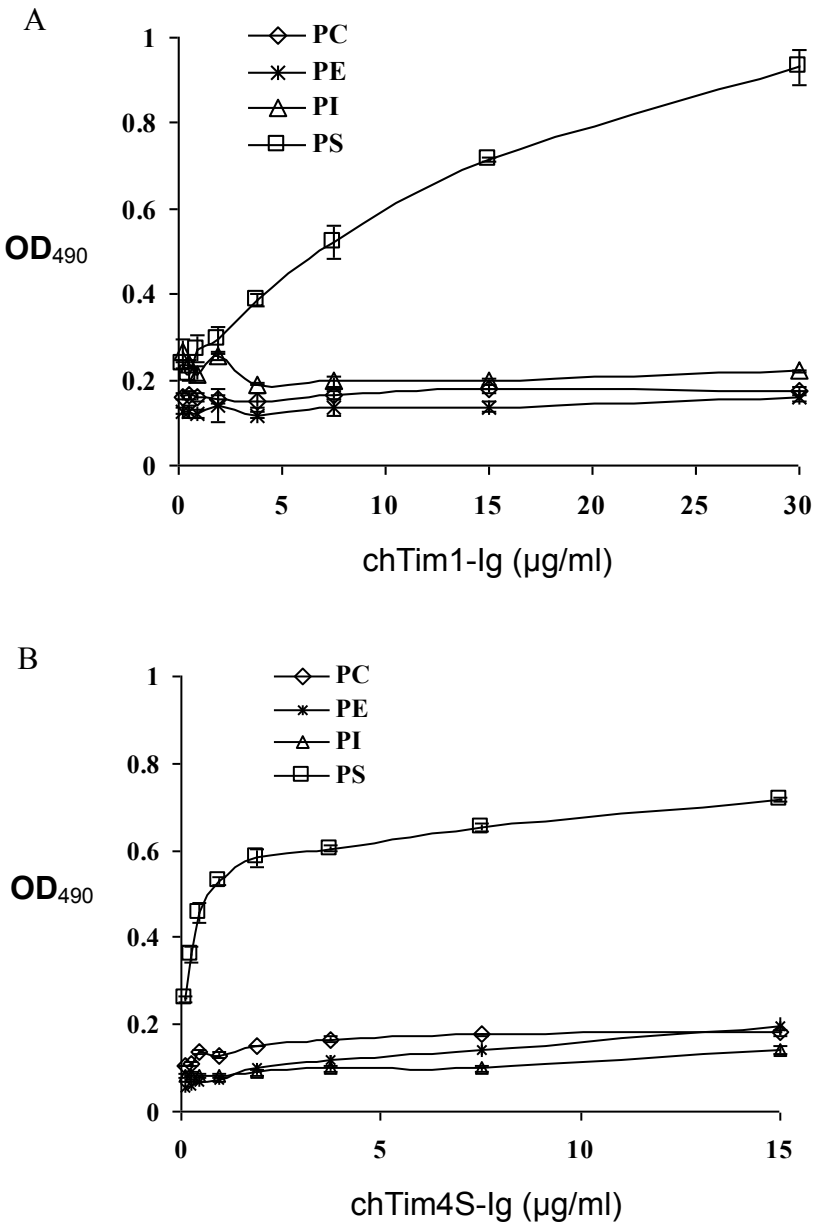


Figure 4.7. Solid phase ELISA assays of interactions of chTim1-Ig and chTim4S-Ig with PS. Phospholipids tested were coated on ELISA plates and purified recombinant (A) chTim1- and (B) chTim4S-extracellular-Ig fusion proteins were 2-fold serially titrated on the plates. The bound Ig fusion proteins were probed with an HRP-conjugated goat anti-human IgG antibody. An OPD substrate was used to detect HRP activity. Error bar represents the standard error in triplicate wells of each dilution. PC = Phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine.

cells or FITC-conjugated annexin V, a receptor of PS, which is normally used to detect PS exposure on dying and dead cells (Kobayashi et al., 2007). The resultant staining was then analysed by flow cytometry, as shown in Figure 4.8. PI staining of ConA-stimulated splenocytes clusters into two clear populations with bright and less bight staining, the former indicating dead cells and latter live cells (Figure 4.8A). PI staining was consistent with analysis of the forward scatter (FSC) versus side scatter (SSC) plot (Figure 4.8B), which is normally used to analyse dead cells in the whole population because condensation of dead cells during apoptosis results in reduced distribution of them in forward scatter and thereby live and dead cells cluster separately in forward and side scatter respectively. The result of annexin V staining was also consistent with PI staining, where an even larger population of ConA-stimulated cells were positively stained by annexin V than that of PI staining, because early apoptotic cells are annexin V positive but PI negative (Kobayashi et al., 2007). The analysis of ConA-stimulated splenocytes suggested that *in vitro* stimulation of chicken splenocytes could induce the cell death.

Therefore, to analyse other potential ligands of Tim4S on the cell surface but avoid any effects of PS, the chTim4S-Ig fusion protein was pre-blocked with PS. PS-blocked chTim4S-Ig was then used to determine if it could bind another ligand on ConA- or rchCD40L-stimulated splenocytes. A rchCTLA4-Ig fusion protein, which binds to its ligands CD80 and CD86 on activated splenocytes, was used as PS-blocking control. The resultant interactions between chTim4 and its ligand(s) were analysed by flow cytometry. As shown in Figure 4.9, the cells were gated into different populations, including dead and live cells, in forward versus side scatter plots. In each gated population, unblocked and PS-blocked chTim4-Ig staining was

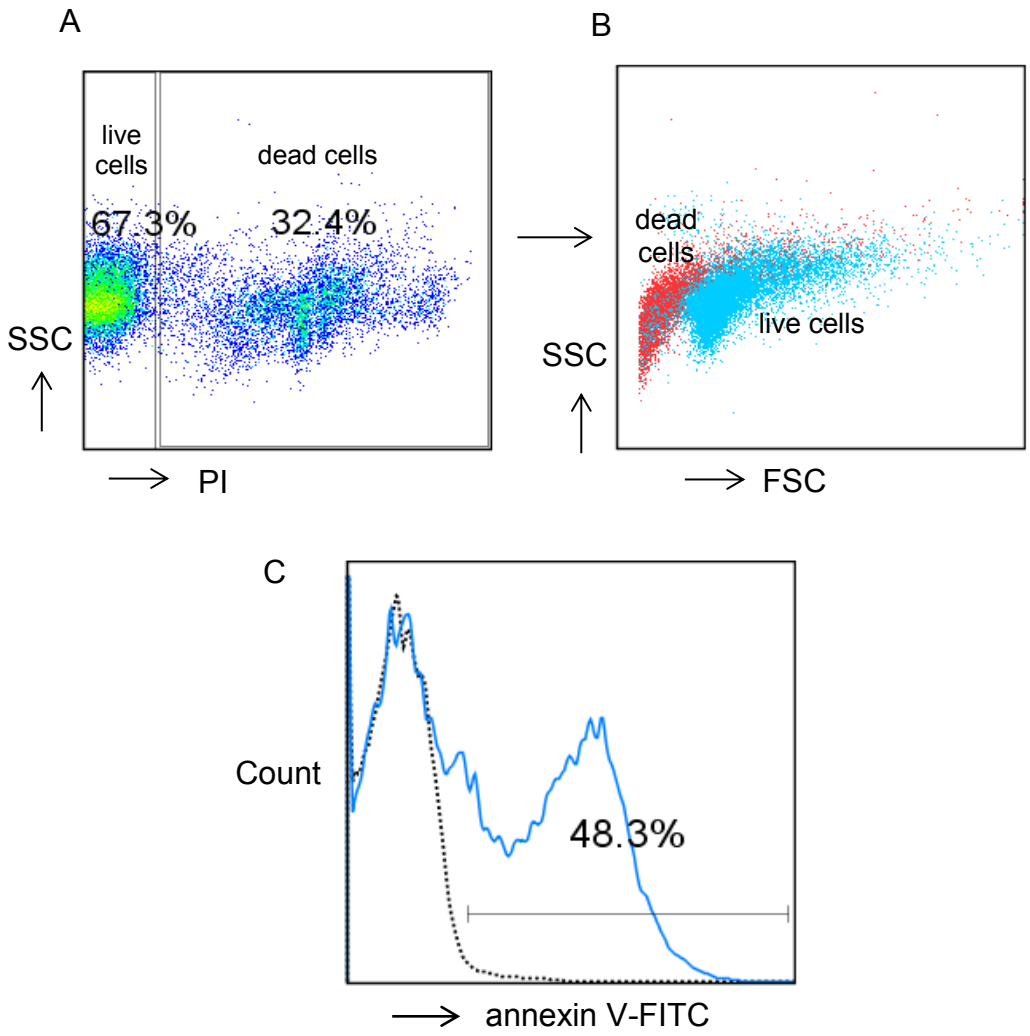


Figure 4.8. PS exposure on apoptotic chicken splenocytes. Splenocytes were cultured with high-dose ConA for 6 h, followed by PI staining. (A) dead cells with bright PI staining and live cells with no PI staining were gated and their percentages calculated; (B) The previously gated dead cells (in red) and live cells (in blue) were analysed by FSC vs SSC plot again, indicating that dead and live cells are separately clustered along SSC or FSC. (C) FITC-labelled annexin V staining of ConA-stimulated splenocytes show in blue, unstained cells as control in dotted line. Marker and percentage show the positive annexin V staining cells.

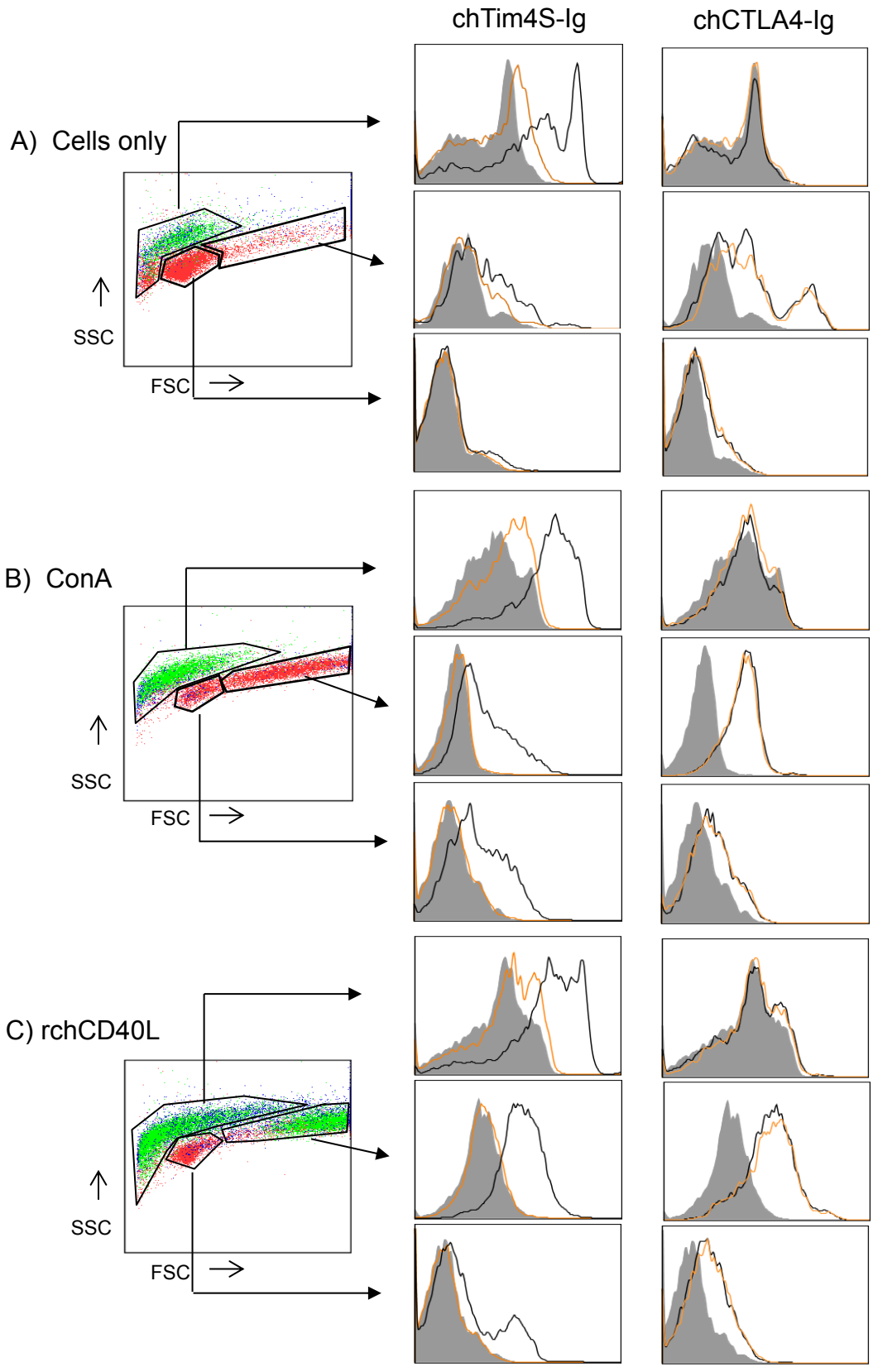

chTim4S-Ig
chCTLA4-Ig
A) Cells only
SSC
FSC
B) ConA
SSC
FSC
C) rchCD40L
SSC
FSC

Figure 4.9. ChTim4S recognises PS exposed on apoptotic splenocytes. Splenocytes were cultured alone (A) or with ConA (B) or rchCD40L (C) for 48 h. In the left-most panels, red represents unstained cells, blue shows annexin V-FITC staining and green shows chTim4S-extracellular-domain IgFc fusion protein staining. The cells were then stained (left column of histograms) with Tim4S-extracellular-domain IgFc fusion protein (black lines), the same protein pre-incubated with PS for 1 h (orange lines) or IgFc fusion protein alone as a negative control (filled histogram). Cells were also stained similarly, but with rchCTLA4-extracellular-domain IgFc fusion protein, as another negative control (right column of histograms). Binding was detected with a rabbit anti-human IgG-FITC polyclonal antibody. Different cell populations were gated by side and forward scatters. For each treatment, the top panel of histograms show dead cells. The middle and bottom panels are live cells; the former were highly stimulated and latter were less stimulated. The flow cytometric results here represent one experiment of four repeats.

compared, plotting overlaid histograms (Figure 4.9).

With unstimulated splenocytes (Figure 4.9A), live cells did not stain with chTim4S (and therefore pre-incubation of chTim4S with PS had no discernible effect). Dead cells, on the other hand, stained strongly with chTim4S and this staining was largely ablated following pre-incubation of chTim4S with PS. For ConA-stimulated cells (Figure 4.9B), both live and dead cells stained with chTim4S (with dead cells staining more strongly) and again, pre-incubation of chTim4S with PS largely ablated this staining. A similar pattern of staining was seen following chCD40L stimulation (Figure 4.9C), in all three populations of cells (dead, live and early apopototic).

Staining with chCTLA4 or chCTLA4 pre-incubated with PS gave essentially identical patterns. For dead cells, in all cases, and unstimulated live cells, these

patterns were the same as those with the negative control IgFc fusion protein. In the live cells following ConA and chCD40L stimulation, staining with chCTLA4 identified a positive population of splenocytes, as would be expected 48 h post-stimulation, but these patterns were not altered by pre-incubation of chCTLA4 with PS.

4.3.7 Phagocytotic activity mediated by chicken Tim molecules

The mammalian Tim molecules enhance phagocytotic activity through recognition of PS exposed on the surface of dead cells (Kobayashi et al., 2007; Miyanishi et al., 2007; Santiago et al., 2007a; 2007b). To investigate if the chicken Tim molecules have the same phagocytotic function, full-length chTim cDNAs in expression vectors were transfected into NIH-3T3 cells to allow the proteins to be overexpressed on the surface of these cells. ConA-induced apoptotic cells, labelled by DAPI, were then mixed with the transfected 3T3 cells to detect chTim-mediated phagocytosis, as described in section 4.2.4. The ultra-violet microscopy results, as shown in Figure 4.10, indicate that 3T3 cells transfected with full-length chTim1, chTim4S and chTim4L phagocytosed more dead cells, with more blue fluorescent dots on the 3T3 cell background imaged with differential interference contrast (DIC), than untransfected 3T3 cells that only had very rare dead cells.

4.3.8 Chicken Tim4S has costimulatory activity on T cells

Murine Tim4 costimulate T cell proliferation mediated by anti-CD3 and -CD28 antibodies *in vitro* (Meyers et al, 2005a). To determine if chicken Tim4S shows similar costimulatory activity, chicken splenocytes were cultured on plates pre-coated with anti-chicken CD3 and/or CD28 antibodies with the addition of

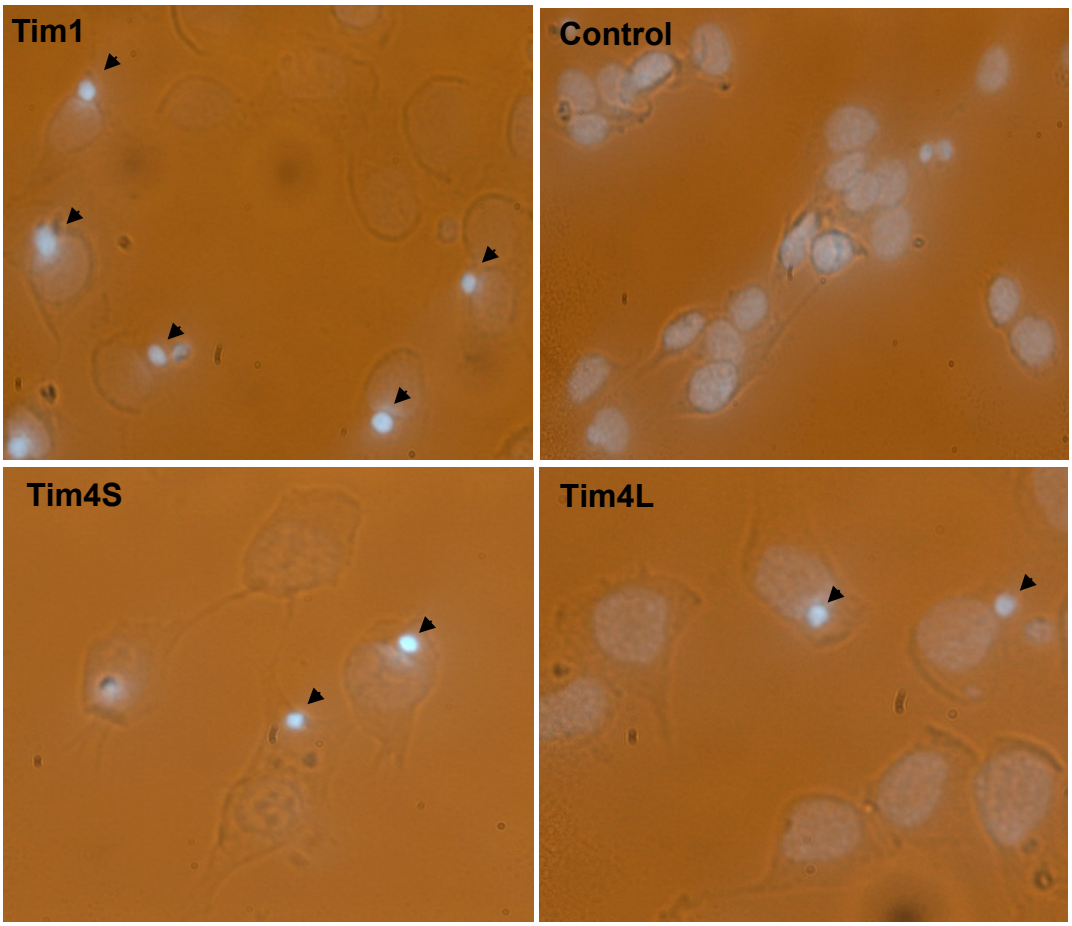


Figure 4.10. Chicken Tim molecules mediate phagocytosis of dead cells. NIH-3T3 cells were transfected with or without full-length chTim1, chTim4S and chTim4L cDNAs. 48 h post-transfection, the high-dose ConA-induced apoptotic cells, labelled by DAPI, were incubated with transfected 3T3 cells for 30 min, and then non-attached cells were washed off. The images were taken through a UV microscope. The bright blue fluorescent dots, shown by arrowheads, show apoptotic cells taken up by the 3T3 cells.

chTim4S-Ig fusion protein in the growth medium. The cells were then pulsed with [^{3}H] thymidine after 48 h, as described in section 4.2.7. As shown in Figure 4.11, the chTim4S-Ig fusion protein or anti-CD3 or -CD28 antibodies alone have no effect on cell proliferation. As expected, anti-CD3 and anti-CD28 stimulated proliferation of splenocytes. The addition of chTim4S-Ig fusion protein stimulated further proliferation.

To prove that costimulation was due to chTim4S and not the Ig tag, an irrelevant Ig fusion protein (human IgG1 Fc) was used as a control in a similar proliferation assay. As shown in Figure 4.12, chTim4S-Ig induced cell proliferation, while the Ig controls had no effect.

4.4 Discussion

The qRT-PCR results revealed that the transcription patterns of the chicken Tim mRNAs are different from those of their mammalian counterparts in the tissues tested. Mammalian Tim1 and Tim4 are described as immune-related molecules, highly expressed in lymphoid organs, but with no detectable expression in non-lymphoid tissues, except for high Tim1 expression in the kidney (Umetsu et al., 2005). Chicken non-immune tissues, on the other hand, also highly express chTim1 and chTim4S. In part, this may be explained by the fact that the chicken immune system does not have lymph nodes. Instead, the chicken develops diffuse lymphoid aggregates in organs (Michael et al., 1974). The chicken spleen contains heterogeneous cell subsets, predominantly resting T and B cells, but also a low number of macrophages and DCs. The chicken thymus mainly contains naïve T cells, and a unique organ of birds, the bursa of Fabricius, predominantly contains naïve B

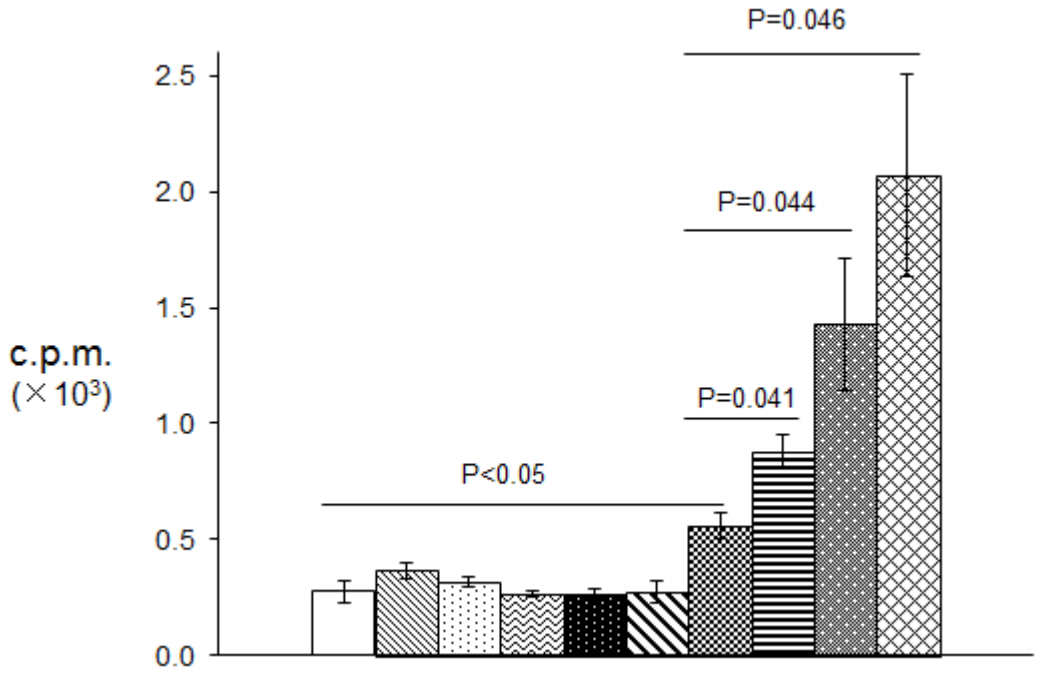


Figure 4.11. Chicken Tim4S-Ig protein costimulates splenocyte proliferation, as measured by [^{3}H] thymidine incorporation. Chicken splenocytes from line 7_2 birds were cultured with or without the addition of Tim4S-Ig fusion protein for 48 h on plates pre-coated with or without anti-chicken CD3 and/or CD28 antibodies. 1, cells only; 2, CD3; 3, CD28; 4, 1 µg/ml protein, 5, 2.5 µg/ml protein; 6, 5 µg/ml protein; 7, CD3+CD28; 8, CD3+CD28+1 µg/ml protein; 9, CD3+CD28+2.5 µg/ml protein; 10, CD3+CD28+5 µg/ml protein. Figure shows means and standard errors in triplicate wells of each treatment. Pairwise comparison analyses, as linked by lines, were carried out by student *t* test and the P values labelled above lines. CD3+CD28 treatment (7) were compared with cells alone (1), or CD3 (2), CD28 (3) alone or different concentration of fusion protein (4-6) with all p values less than 0.05. The results represent one experiment of three repeats.

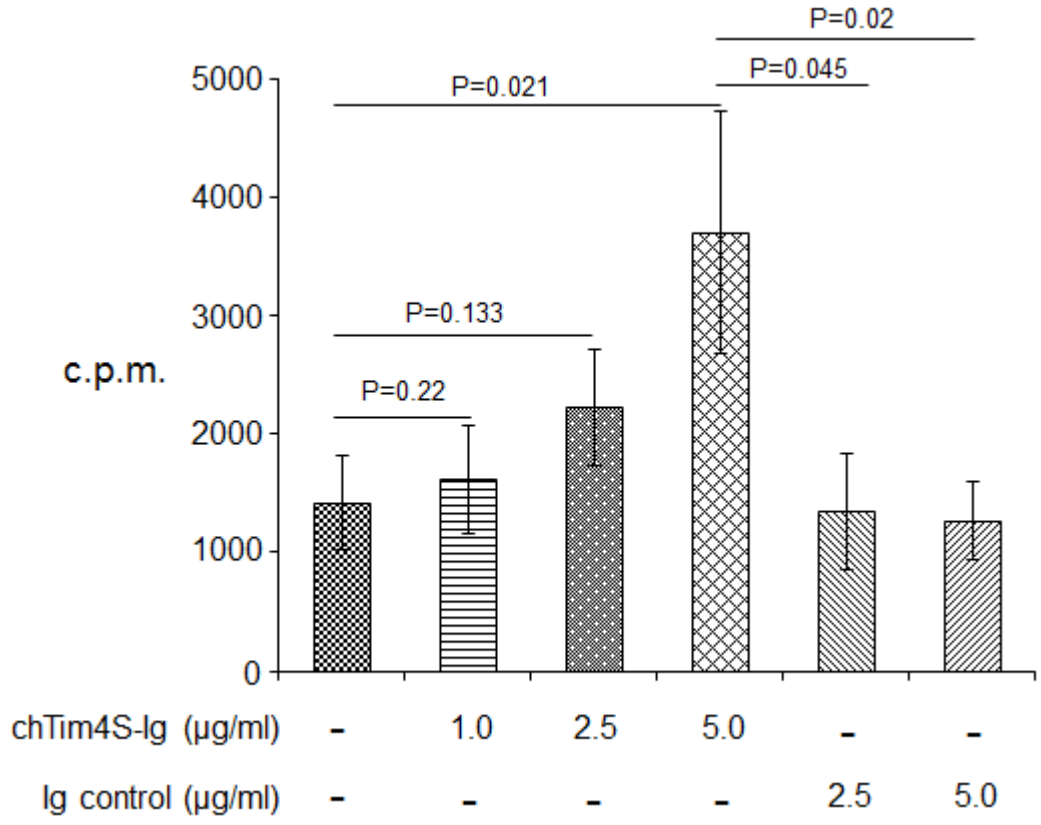


Figure 4.12. Re-analysis of chTim4S-Ig protein bioactivity in costimulation of splenocyte proliferation as measured by [^{3}H] thymidine incorporation. Chicken splenocytes were cultured with or without addition of Tim4S-Ig fusion protein for 48 h, with an irrelevant Ig protein (human IgG Fc) as negative control, on plates pre-coated with anti-chicken CD3 and CD28 antibodies. Figure shows the means and standard errors in triplicate wells of each treatment. Pairwise comparison analyses, as linked by lines, were carried out by student *t* test and P values labelled above lines. The results represent one experiment of two repeats.

cells. The mRNA of exons 3 and 4-containing chTim4 isoforms (chTim4eL, chTim4L and chTim4eS) can be detected in thymus (Figure 4.1), suggesting that they are expressed by naive T cells but at very low level. Their mRNAs were also measured at similar expression levels in the bursa of Fabricius and spleen (Figure 4.1), suggesting B cells may also express these novel chTim4 isoforms.

$CD4^+$ and $CD8^+$ T cells and DCs expressed low levels of the chTim mRNAs, but they were abundantly present in $TCR1^+$ T cells, B cells and macrophages (Figure 4.2). The mRNA expression patterns of chTim1 on immune cells is consistent with that of mammalian Tim1, at low or undetectable levels in resting or naïve splenic $CD4^+$ and $CD8^+$ T cells (Mesri et al., 2006; Khademi et al., 2004), but constitutively expressed in splenic B cells (Sizing et al., 2007) and DCs (Xiao et al., 2011). By contrast, chTim1 is expressed at highest levels in chicken $TCR1^+$ T and $Bu\text{-}1^+$ B cells, suggesting it may have more important roles in these cell subsets than in other cells. Blood monocyte-derived chicken macrophages also expressed chTim1 mRNA at high levels. Whether this expression pattern reflects a role of chTim1 on macrophages to mediate phagocytosis of dead cells requires further elucidation.

In murine splenic cell subsets, Tim4 mRNA is highly expressed in macrophages ($CD11b^+$), DCs ($CD11c^+$), at low level in splenic B cells ($B220^+$), but has very low or undetectable expression in murine T cells ($CD3^+$) (Meyers et al., 2005a). Murine Tim4 mRNA is also expressed in peritoneal macrophages and LPS-matured murine DC generated from bone marrow with Flt3L, but not in immature DC (Miyanishi et al, 2007; Meyers et al., 2005a). Chicken Tim4 (predominant chTim4S) has a similar mRNA expression pattern to that of mammalian Tim4 - low expression levels in splenic $CD4^+$ and $CD8^+$ T cells, but high expression levels in

macrophages (Figure 4.2B). Immature bone marrow-derived DCs induced with rchGM-CSF and rchIL-4 also expressed chTim4 mRNA, but at low levels, and there was no obvious variation in chTim4 mRNA expression between immature and mature DCs (Figure 4.2B, C), different to what was seen in mice, possibly reflecting the different culture methods. Consistent with qRT-PCR analysis in the tissue panel (Figure 4.1C), chTim4 mRNA isoforms containing exons 3 and 4 (chTim4eL, chTim4L and chTim4eS) were also expressed by different chicken immune cells from the spleen at low levels (Figure 4.2C). Interestingly, these chTim4 isoforms were not expressed by blood monocyte-derived macrophages under any conditions; by contrast, chTim4S was expressed at high levels in these cells and expression levels varied with different stimulation, such as downregulation with LPS stimulation, but upregulation with rchCD40L stimulation (Figure 4.2B), suggesting chTim4S is the predominant isoform present in blood monocytes or monocyte-derived macrophages. Splenic Bu-1^+ cells had the highest levels of chTim4 expression, suggesting that chTim4 may have a more important role in B cells than in other APCs. The expression of chTim4 at the protein level in chicken APCs will be further studied using mAb immunostaining in Chapter 6.

It is not clear why splenic TCR1^+ cells also express chTim4 and chTim1 mRNA at high levels (Figure 4.2). Chicken splenic TCR1^+ cells are γδ T cells which tissue-specifically co-express CD4 and CD8. In the thymus and blood chicken γδ T cells are $CD4^-CD8^-$. When they migrate into the spleen and intestine, two thirds of the γδ T cells begin to express CD8 (Chen et al., 1994; Erf et al., 1998). Although TCR1^+ $CD8^+$ T cells are abundantly present among splenocytes, the splenic $CD8^+$ T cells had low levels expression of chTim1 and chTim4 (Figure 4.2), suggesting that

high level expression may be from $TCR1^{+}CD8^{-}$ cells. Chicken $TCR2^{+}$ and $TCR3^{+}$ splenocytes are αβ T cells, but expression of chTim1 and chTim4 mRNAs could not be detected in these T cells (Figure 4.2), suggesting they may not be involved in chTim4-associated functions, at least in naïve or resting stages.

Splenic $TCR2^{+}$ T cells did not express chTim4 mRNA, which was also demonstrated by RT-PCR in a new panel of sorted splenic subsets (Figure 4.3). Consistently, $CD4^{+}$ T cells expressed undetectable levels of chTim4, and other T cell subsets ($CD8^{+}$ and $TCR1^{+}$), B cells ($Bu\text{-}1^{+}$) and macrophages ($KUL01^{+}$) in the spleen expressed high levels of chTim4S (Figure 4.3). By contrast, chTim4L was expressed only by cultured and activated splenic splenocytes (Figure 4.3). Both qRT-PCR and RT-PCR analysis consistently indicated that chTim4S is predominantly expressed in normal immune tissues and cells (Figures 4.2-4.3), suggesting chTim4S is a constitutive form of chTim4 and may be homologous to mammalian Tim4.

Chicken Tim ligands expressed on splenocytes were detected with chTim-Ig fusion proteins (Figure 4.4). The chTim1- or chTim4S-IgV domains contain the ligand binding sites as they bound but the mucin domains have no detectable binding sites, as they did not bind. However, IgV + mucin domain bound more strongly than the IgV domains alone, suggesting that the chTim IgV domains play critical roles in binding to the receptor(s), whereas the mucin domains have no specific ligand but act to enhance the binding affinity.

The chTim4S-Ig fusion protein was also used to study expression of its ligand on chicken splenocytes after stimulation. Flow cytometric analysis (Figure 4.5) revealed dramatic upregulation of chTim4S ligands on ConA- or rchCD40L-

stimulated splenocytes. Similar results were also seen for murine Tim4, where Tim4 ligand expression on activated splenic cells led to the hypothesis that Tim1 is a natural ligand of Tim4, which was eventually demonstrated with studies showing that Tim4-Ig fusion protein bound to full-length Tim1-transfected CHO cells and that the Tim4-Tim1 interaction can be blocked with an anti-Tim1 mAb (Meyers et al., 2005a).

Phosphatidylserine (PS) was also found to be a ligand of murine Tim4. The crystal structure of the murine Tim4-IgV domain identified a metal-ion-dependent ligand binding site (MILIBS). PS penetrates into the MILIBS and co-ordinates with the metal ion (Santiago et al., 2007a). Tim4 bound to PS mediated uptake of apoptotic cells, but mutations in this site eliminated PS binding and phagocytosis (Kobyashi et al., 2007). The chicken Tim1 and Tim4 molecules also contain this conserved PS binding site, which suggests that the chicken Tim molecules may play roles in the phagocytosis of dead cells. To test this hypothesis, the chTim1- and chTim4S-Ig fusion proteins were used to hybridise with different phospholipids on a solid phase. The protein-phospholipid overlay assays (Figures 4.6 and 4.7) demonstrated that chTim fusion proteins specifically bind to PS, but not to other phospholipids. In quiescent cells, PS is restricted to the inner plasma-membrane leaflet. But apoptotic, injured, infected, senescent or necrotic factors can cause PS externalisation to the outer leaflet. Activation stimuli can also induce PS exposure on different immune cells, such as stimulated B cells after anti-IgM cross-linking (Dillon et al., 2000), T cells stimulated with protein melan-A, known as melanoma antigen recognized by T cells (Fischer et al., 2006) and mitogen-activated master cells (Smrz et al., 2007). PS exposure strongly depends on the amount of presented

antigen. This raised the question, which ligand is upregulated on stimulated chicken splenocytes, chTim1, PS or both? To identify the potential ligand but avoid the PS effect, chTim4S-Ig fusion protein was pre-incubated with PS and then added to stimulate chicken splenocytes for flow cytometric analysis again. As per the previous observations, the chTim4S-Ig protein bound to most dead and activated cells. After PS blocking there was no detectable binding of chTim4S-Ig to cells, suggesting PS is predominantly exposed on activated splenic cell surfaces, whereas any other potential ligand may be expressed at very low levels, or PS blocking binding, or PS causes conformational change.

Hoffmann et al. (2001) proposed a two-step model of phagocytosis, where in first step apoptotic cells and phagocytes are bridged through interaction between PS and its receptors; for example, Tim4 catches apoptotic cells by recognizing PS. A putative co-receptor (for signal transduction) is coupled to Tim4 to activate the integrin $\alpha_v\beta3$ complex to initiate uptake, in which milk fat globule epidermal growth factor VIII (MFG-E8) binds to PS on apoptotic cells and to the activated integrin $\alpha v\beta3$ complex on the phagocytes. The signal from the integrin $\alpha v\beta3$ complex then activates the macrophages' internalisation of apoptotic cells into lysosomes. This final step is mediated by Rac1, Rab5, and other, as-yet-unidentified molecules.

To determine if this model applies in the chicken, it is essential to address if the interaction between chicken Tim molecules and PS enhances phagocytosis. An *in vitro* phagocytosis model (well-studied in mammalian Tim molecules), NIH-3T3 cells overexpressing chTim protein as phagocytes, was used to detect chTim molecule-mediated phagocytosis. As analysed by flow cytometry after annexin V

and PI staining (Figure 4.8), the apoptotic chicken splenocytes exposed PS on their cell surface after high-dose ConA stimulation. When these apoptotic cells were mixed with 3T3 cells transfected with full-length cDNAs of chTim1, chTim4S and chTim4L (Figure 4.10), the transfected 3T3 cells phagocytosed more apoptotic cells than untransfected cells. Although it is not known to what extent the previous qRT-PCR data for different chicken cells reflects their expression at the protein level, the binding of chTim1 and chTim4S to PS suggests they play crucial roles in the phagocytosis of dead cells by macrophages and DCs. qRT-PCR indicated that the chTim mRNAs were highly expressed by γδ T cells ($TCR1^+$). The biological function of these cells is unclear, but they are clearly capable of cytotoxic activity *in vitro* (Chen et al., 1994). The expression of chTim molecules on chicken γδ T cells may help capture apoptotic or infected cells through recognition of PS, and facilitate cytotoxic activity.

Administration of murine Tim4-Ig fusion protein *in vivo* with antigen produces increased basal T cell proliferation and enhanced production of IL-2 and IFN-γ (Meyers et al., 2005a). Several *in vitro* studies also indicate that Tim4-Ig can play bilateral roles in T cell proliferation. In the presence of anti-CD3 and -CD28 antibodies for pre-activation of naïve T cells, Tim4-Ig induced dramatically higher levels of proliferation and cytokine production than those stimulated with a control protein, as well as an increased phosphorylation of Tim1. Tim4 was therefore defined as a costimulatory molecule that promotes T cell expansion and survival by cross-linking Tim1 on activated T cells (Meyers et al., 2005a, Rodriguez-Manzanet et al., 2008). By contrast, murine Tim4-Ig inhibited the proliferation of naïve T cells, on which there is very low or no Tim1 expression. It was supposed that Tim4-Ig

suppressed the naïve T cell proliferation by binding to an unknown ligand (Mizui et al., 2008). The chicken Tim4S-Ig fusion protein induced splenocyte proliferation in the presence of anti-chicken CD3 and CD28 antibodies (Figures 4.11 and 4.12). Although it is not determined which T cell subset(s) proliferated, this result suggests that the chicken Tim4S molecule has a function in the regulation of T cell immune responses.

It has been demonstrated in this Chapter that chTim molecules have a potent physiological role in the phagocytosis of apoptotic cells by their interaction with PS. The chTim4S-Ig fusion protein also showed costimulatory activity in enhancement of splenocyte proliferation. To determine in more detail how these roles are fulfilled, it is essential to know the expression patterns of the chTim molecules on chicken primary cells. Therefore, anti-chTim mAbs will be generated and characterised in Chapter 5, and specific expression of chTim proteins on chicken immune cells will be further investigated with these mAbs in Chapter 6.

Chapter 5. Generation and characterisation of anti-chicken Tim antibodies.

5.1 Introduction

In mammals, Tim1 has a wide variety of physiological and pathological functions, such as being a marker of acute kidney injury (Kim-1) reflecting ischemic kidney injury (Ichimura et al., 1998), and a receptor for the hepatitis A virus (HAVCR-1) associated with its invasion (Silberstein et al., 2003). Allergy, atopic and autoimmune diseases also have a strong association between expression levels of Tim1 and disease development (Meyers et al., 2005b). Tim1 is preferentially expressed on activated Th2 T cells, but not on naïve T cells, acting as a positive regulator in Th2 cell immune responses (Umetsu et al., 2005; de Souza et al., 2005). Cross-linking Tim1 using an agonistic anti-Tim1 antibody (3B3) can costimulate Th2 cells to proliferate and increase IL-4 production in the presence of anti-CD3 and -CD28 antibodies (Umetsu et al., 2005). The costimulatory effects of Tim1 on T cells suggest the possibility of targeting Tim1 to develop novel prophylactic and/or therapeutic vaccination strategies. An anti-Tim1 mAb used as an adjuvant with an influenza vaccine enhanced antigen-specific lymphocyte proliferation, IFN-γ production and cross-virus strain reactivity (Soo Hoo et al., 2006). However, cross-linking of Tim1 can also have inhibitory activities. Another Tim1-specific mAb (RMT1-10), sharing an overlapping epitope in the IgV domain with 3B3 but with low avidity, decreased antigen-specific $CD4^+$ T cell expansion and production of IFN-γ and IL-17 by these cells (Xiao et al., 2007). In treatment of experimental autoimmune encephalomyelitis (EAE), a mouse model of human multiple sclerosis

(MS), administration of RMT1-10 ameliorated antigen-induced EAE, suggesting an antagonistic anti-Tim1 antibody may have beneficial effects against human MS. However, in the same experiment, the previously agonistic mAb 3B3 exacerbated EAE, suggesting the epitope and strength of antibody should be carefully considered if anti-Tim1 antibodies are to be used for immunotherapy (Xiao et al., 2007). Activating/blocking activities of anti-Tim1 mAbs were also independently demonstrated by another group in a study of antigen-induced asthma (Sizing et al., 2007). Two anti-Tim1 antibodies, against different epitopes in the IgV (4A2.2) and mucin domains (3A2.5), had inhibitory activity on antigen-induced lung inflammation and caused a large reduction in Th2 cytokine production (Seizing et al., 2007). However, in the same experiment, *in vivo* administration of another two anti-Tim1 antibodies (5D1.1 and 1H8.2), both which recognise epitopes which mapped to the mucin domain, significantly enhanced lung responses to antigens and increased expression of Th2 cytokines in *ex vivo* re-stimulation (Seizing et al., 2007). All this evidence suggests that Tim1 plays reciprocal roles in the regulation of Th2 immune responses.

In attempts to identify a Th1-specific cell surface protein, Th1 cell clones and lines were used to immunise mice. Two mAbs from 20,000 tested selectively stained Th1 but not Th2 cells. A target antigen, Tim3, was eventually identified as being specifically expressed on the surface of Th1 cells (Monney et al., 2002). Tim3 plays a negative role in the regulation of Th1 cell responses. *In vitro* addition of a Tim3 ligand, galectin-9, induced Tim3-dependent Th1 cell death but not that of Th2 cells (Zhu et al., 2005). *In vivo* administration of galectin-9 also downregulated Th1 immune responses (Zhu et al., 2005). Tim3 is also expressed on DCs and

macrophages and synergises with TLRs to regulate innate immunity (Anderson et al., 2007). Chronic HIV and HCV co-infections impaired Th1 and cytotoxic $CD8^+$ T cell immune responses with high level expression of Tim3 and programmed cell death 1 (PD-1) on the cell surface. However, *ex vivo* addition of an anti-Tim3 mAb, which functionally blocks the interaction between Tim3 and its ligand, to PBMC cells isolated from these infected patients, rescued Th1 and cytotoxic $CD8^+$ T cell immune responses and IFN-γ production (Vali et al., 2010).

In a library of hamster mAbs generated against mouse peritoneal macrophages, a single mAb strongly inhibited the PS-dependent engulfment of apoptotic cells, and this antigen was eventually identified as the murine Tim4 molecule. It has been well-documented that Tim4 is a receptor for PS and plays a role in the enhancement of dead cell clearance (Miyanishi et al., 2007). Tim4 is also a natural ligand of Tim1. *Ex vivo* administration of a Tim4-Ig fusion protein in the presence of anti-CD3 and -CD28 mAbs can stimulate T cell proliferation, suggesting a costimulatory activity derived from the Tim4-Tim1 interaction (Meyers et al., 2005a). However, Tim4-Ig fusion proteins can also bind to naïve T cells, which do not express Tim1, and inhibit activation of these cells, suggesting an unkown ligand of Tim4 may exist on naïve T cells. On pre-activated T cells, this Tim4-Ig inhibitory activity disappears (Mizui et al., 2008; Cao et al., 2011).

In an attempt to generate anti-chicken Tim mAbs, mice were immunised with Ig-tagged chTim fusion proteins; the splenocytes were subsequently isolated and fused with a partner cell line to generate immortal hybridomas for producing antigen-specific antibodies. The specificity of the anti-chTim mAbs was then intensively

examined with different methods, including ELISA, immunostaining of transfected COS-7 and CHO cells, western blot, etc.

5.2 Materials and Methods

5.2.1 Immunization of mice

Two Biozzi strain mice were immunized with a chTim1-extracellular-Ig fusion protein for generation of anti-Tim1 antibodies and three BALB/C strain mice with a chTim4S-extracellular-Ig fusion protein for anti-Tim4 antibodies. Two Biozzi strain mice were also immunised with a chTim4L-hinge-Ig fusion protein to generate mAbs against novel Tim4 isoforms, including potentially chTim4eL, chTim4L and chTim4eS. All mice were pre-bled on day 0 from the tail vein by cutting a small nick and collecting blood for serum. On day 1, each mouse was primarily immunised by subcutaneous injection of 50 μg purified Ig fusion protein which was emulsified with adjuvant, TiterMax gold (Invitrogen). A first boost was given on day 21 by the same procedure as the primary immunisation. On day 28, the mice were bled from the tail vein for serum. A second boost was given on day 35. One week later (on day 42), the mice were bled from the tail vein again for serum. A final boost was performed on day 50 by intraperitoneal (ip) injection of 50 μg purified Ig protein without any adjuvant.

5.2.2 Evaluation of immune response by ELISA

The immune responses of the immunised mice against the chTim1 and chTim4S Ig fusion proteins were tested by a standard indirect ELISA. Briefly, purified chTim1- and chTim4S-His6 proteins (mutually as controls) were coated on

ELISA plates (section 2.6.1). After blocking the plates with 1% casein/PBS, the sera, including pre-immunisation and immunization sera, were serially titrated on the plates, followed by probing with HRP-conjugated goat anti-mouse IgG. The HRP activity was detected with OPD substrate.

A capture ELISA assay was applied to analyse immune responses against the chTim4L-hinge-Ig fusion protein, as described in section 2.6.1. As shown in Figure 5.1, to eliminate any background caused by anti-Ig antibodies in the anti-chTim4L-hinge-Ig sera, a competitive ELISA assay was also used to evaluate immune responses. An ELISA plate was coated with chTim4L-hinge-Ig fusion protein or irrelevant Ig protein (rchCD86-Ig) as negative control. The sera from day 42 were diluted at 1:150 in PBS. The serum solution (100 µl) was then mixed with 10 µg irrelevant Ig fusion protein (rchCD86-Ig) or PBS and incubated at room temperature for 1 h to allow blockade of anti-Ig responses. The sera were then serially titrated on plates. The rest of the ELISA procedure was as described previously.

5.2.3 Fusion of immunized mouse spleen cells with partner cells, SP2/0

Two days before fusion, mouse peritoneal macrophages as feeder cells were grown on flat-bottomed 96-well plates in complete RPMI 1640 medium plus 1× HAT reagent (hypoxanthine/aminopterin/thymidine, Life Technologies) at 37ºC, 5% CO_2. One day before fusion, cultured SP2/0 cells were split to ensure that cells were in the exponential phase of growth at the time of fusion. On fusion day, the SP2/0 cells were split, pelleted and resuspended in serum-free RPMI 1640.

The spleens from the immunised mice were collected and single cells were flushed out with a 10 ml syringe filled with serum-free RPMI 1640 and with a 26G

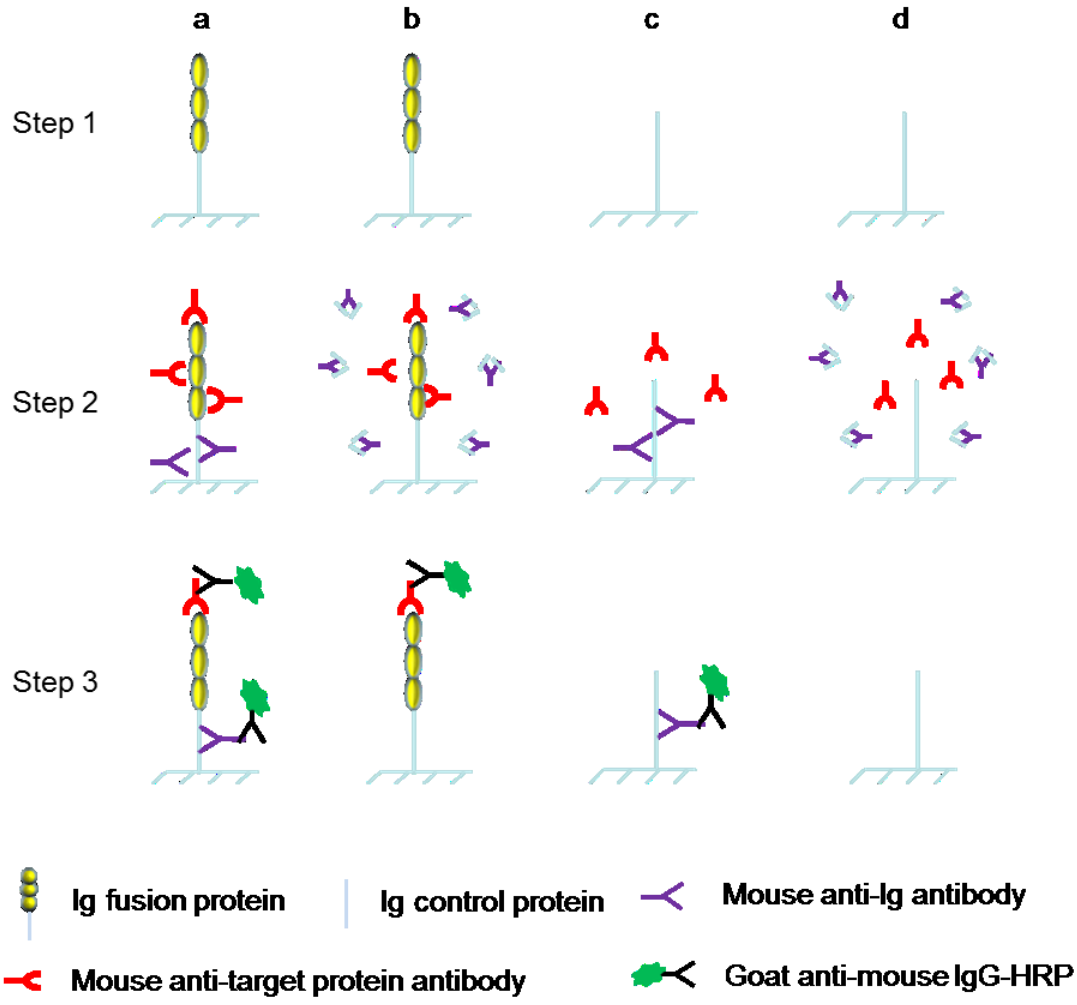


Figure 5.1. Competitive ELISA assay. Ig fusion protein (a, b) or Ig control protein (c, d) were coated on solid phase in step one. Sera (a, c) or Ig pre-blocking sera, rchCD86-Ig (b, d) were added onto the plate in step 2, follow by HRP-labelled secondary antibody in step 3. Finally, the HRP activity was detected by an OPD substrate.

needle attached. The flushing was repeated at various points along the spleen until the bulk of the contents were removed. The cell suspension was transferred to a new Universal and centrifuged at 1,100 *g* for 5 min. The cell pellet was resuspended in 5 ml serum-free RPMI 1640. Prior to counting the cells, cell suspension (100 µl) was mixed with 4.9 ml 0.85% NH_4Cl and incubated for 2 min at room temperature to swell B cells and lyse red blood cells. SP2/0 cells were then mixed with the spleen cells in a new Universal at a ratio of 1:5. The combined cells were pelleted as before and the supernatant discarded. To fuse the cells, 1 ml pre-warmed polyethylene glycol (PEG) (Roche) was slowly added to the cells, followed by the addition of 10 ml pre-warmed serum-free RPMI 1640 to dilute the PEG. The fused cells were pelleted again as before and resuspended in HAT selective medium (complete RPMI 1640 growth medium plus 1× HAT). The cell density was adjusted to 1×10^6 cells/ml and the cell suspension (100 µl/well), including unfused spleen cells as a control, was then dispensed into the above prepared macrophage plates and returned to the 37ºC, 5% CO_2 incubator.

5.2.4 Positive hybridoma selection

Control wells were tested for background activity after 5 days growth in 96-well plates. The cells were then refed by replacing 0.1 ml medium from each well with 0.2 ml HAT selective medium.

The main screenings for positive clones were carried out on day 10. ChTim4L-hinge-Ig fusion protein was used in an indirect ELISA to screen hybridomas for anti-chTim4L-hinge antibodies as described in section 2.6.1. To diminish the effect of Ig responses, the positive wells were re-screened by a

competitive ELISA (section 5.2.2). The hybridoma supernatant, 10 µl per sample, was diluted in 90 µl 1% casein/PBS, and then blocked with 1 µg (10 µl) irrelevant Ig fusion protein (rchCD86-Ig) for 1 h at room temperature. The supernatants (50 µl) were then added to test and control wells on the plates for ELISA assays.

Hybridomas which produced anti-chTim1 or -chTim4S antibodies were screened using an indirect ELISA (section 5.2.2), in which His6-tagged chTim1 or chTim4S proteins were used as specific antigens in the corresponding assays.

5.2.5 Subcloning monoclonal hybridoma

The positive hybridomas were split from 96-well plates and grown up in 24-well plates with pre-grown mouse peritoneal macrophages. After 3-5 days growth, when the cells were 60-70% confluent, the supernatants were collected for further testing as before. The hybridomas were then ranked in order depending on the test results and several of the best hybridomas were chosen for subcloning using a limiting dilution method. In brief, the cells were split, counted and diluted at 2×10^3 cells/ml in 10 ml HT medium, complete RPMI 1640 medium plus 1× HT reagent (hypoxanthine/thymidine, Sigma-Aldrich), in a Universal. This cell suspension (1 ml) was further diluted in 9 ml HT medium, from which 3 ml of cell suspension were further diluted in 7 ml HT medium. On a 96-well plate with pre-grown mouse peritoneal macrophages, cell suspension from the first dilution was plated out on half of a plate at 100 µl/well, and 100 µl per well of cell suspension from the second dilution on the other half of the plate. The cell suspension from the third dilution was dispensed on a new macrophage plate at 100 µl/well. The cells were cultured at 37ºC, 5% CO_2 for 5-7 days. The clones in each well were scored under a microscope

and the wells containing a single clone identified. Two days later, the hybridamas were screened by ELISA. Several wells with the highest ELISA readings containing single clones were split and grown up in 24-well plates. After a further ELISA, the best hybridomas were chosen for a second round of subcloning.

5.2.6 Labelling of monoclonal antibodies

After double subclonings, the monoclonal hybridomas were grown up at a large scale, the tissue culture supernatants harvested and the mAbs purified as described in section 2.9.

The purified mAbs were biotinylated using Sulfo-NHS-LC-Biotin reagent (Thermo Scientific) according to the manufacturer's instructions. Briefly, the mAbs were adjusted to 2 mg/ml by either concentration with 100 kDa Amicon Ultra centrifugal filter units (Sigma-Aldrich) or dilution with PBS. Immediately before use, 2.3 mg Sulfo-NHS-LC-biotin reagent were dissolved in 500 µl DMSO to make a 10 mM biotin solution. For each labelling reaction, biotin solution (27 µl) was added to 1 ml (2 mg/ml) mAb solution, mixed well and incubated at room temperature for 30 min. The labelled mAbs were then dialysed in Slide-A-lyzer dialysis cassettes (30 kDa molecular weight cut-off, Thermo Scientific) against PBS to remove uncoupled biotin reagent and DMSO. Finally, the biotinylated mAbs were filtered through a syringe filter with 0.2 µm pore size.

The purified mAbs were also coupled with a fluorescent dye using an Alexa fluor 647 monoclonal antibody labelling kit (Invitrogen) as per the manufacturer's instructions. Briefly, 10 µl 1 M sodium bicarbonate were added to 100 µl antibody solution (100 µg) This mix (100 µl) was added to a reactive dye, mixed well and

incubated for 1 h at room temperature to label the antibody. A spin column was prepared by filling it with 1.5 ml resin and excessive liquid removed by centrifugation at 1,100 *g* for 3 min. To remove unlabelled dye from labelled antibody, the antibody/dye mixture was loaded onto the column and centrifuged at 1,100 *g* for 3 min. Flowthough was collected containing the successfully labelled antibody and stored at 4ºC.

5.2.7 Antibody epitope mapping

The epitopes recognised by the anti-chTim mAbs were mapped to different domains using capture ELISA assays as described in section 2.6.1. Briefly, chTim-IgV, -mucin or -extracellular domain Ig fusion proteins, transiently expressed by COS-7 cells, were captured on ELISA plates pre-coated with unlabelled goat anti-human IgG antibody. The primary mAbs against chTim1 or chTim4S were then serially titrated on the plates. A goat anti-mouse IgG-HRP antibody was used to detect the bound primary antibody and the HRP activity was detected with an OPD substrate.

The binding sites of anti-Tim1 mAbs were also tested by a competitive ELISA. Briefly, the ELISA plate was coated with chTim1-His6 protein (section 2.6.1) and blocked with 1% casein/PBS as before. Unlabelled anti-chTim1 antibodies or PBS as control were added to the plate and incubated for 1 h. After washing the plate, biotinylated anti-chTim1 mAbs or a biotinylated isotype control mAb (ILA12), 1 µg/ml, 50 µl/well, were then added to the plate. HRP-conjugated streptavidin (1:1000 dilution) was used to detect the bound biotinylated mAbs. OPD substrate was used to detect the HRP activity.

5.2.8 Transfected COS-7 cell lysates

COS-7 cells were transfected with full-length chTim1, chTim4S and chTim4L cDNAs cloned in pCIneo using a Gene Pulser Xcell electrophoration system (Bio-Rad), as described in section 2.7.2. Viable transfected cells (10^6) were seeded in a T75 tissue culture flask and cultured for 48 h. The cells were removed from the flasks with cell scrapers (Sigma-Aldrich). The cells were pelleted and washed twice with cold PBS. To lyse the cells, 2×10^6 cells were resuspened in 100 µl PBS and mixed thoroughly with an equal volume of 2× SDS-PAGE reducing sample buffer. The cell lysates were then immediately heated at 95ºC for 5 min and stored at -20ºC.

5.2.9 Whole cell ELISA

Full-length chTim1, chTim4S and chTim4L cDNAs were stably transfected into CHO cells and single cell clones were grown up in 96-well plates as described in section 3.2.3. The single cell clones were then split and equally reseeded into two new 96-well plates, one clone/well/plate. When the cells were fully confluent, the clones with high-level protein expression were screened with a whole cell ELISA assay. Briefly, supernatant was removed from the plates and the cell layers washed twice with PBS, 200 µl/well. To fix the cells, 100% ethanol was added to the cells, 100 µl/well, and incubated for 10 min at room temperature. The plate was washed twice with PBS and then blocked with 1% casein/PBS (100 µl/well) for 1 h. The primary mAbs, GG9 (anti-chTim1), JH9 (anti-chTim4S) and IE12 (anti-chTim4L-hinge), were added to the corresponding plates. The bound primary antibodies were detected by biotinylated goat anti-mouse IgG antibody (Amersham Bioscience). The

biotin was then detected using an ABC kit (GE Healthcare) as per the manufacturer's instructions. One drop of solution A was diluted in 10 ml of PBS and then mixed with one drop of solution B, incubating for 30 min to form the biotin-avidin complex (avidin is conjugated with HRP). This complex, 50 µl/well, was added to plates and incubated for 30 min. OPD substrate solution was used to test the HRP bioactivity. The optical density was read at a wavelength of 490 nm. Six positive clones from each transfection were chosen to grow up in 24-well plates and retested again by the whole cell ELISA assay. The best clone from each transfection was then grown up for testing anti-chTim mAbs in flow cytometric analysis.

5.3 Results

5.3.1 Mice immune responses post-immunisation with chTim-Ig proteins

To generate monoclonal antibodies against chTim1 or chTim4, purified Ig-fusion proteins, containing the whole extracellular domains of chTim1 or chTim4S, were used to immunise mice. To generate antibodies that would distinguish novel chTim4 isoforms (chTim4eL, chTim4L and chTim4eS) from chTim4S, an Ig-tagged fusion protein containing the hinge (encoded by exons 3 and 4) between the two IgV domains of chTim4L (chTim4L-hinge-Ig) was generated and used to immunise mice. The mouse sera were sampled before and between immunisations. ELISA assays were used to analyse the serological immune responses against the corresponding antigens.

A His6-tagged chTim1 protein was used as antigen to detect chTim1-specific antibodies in sera by an indirect ELISA (section 5.2.2). As showed in Figure 5.2,

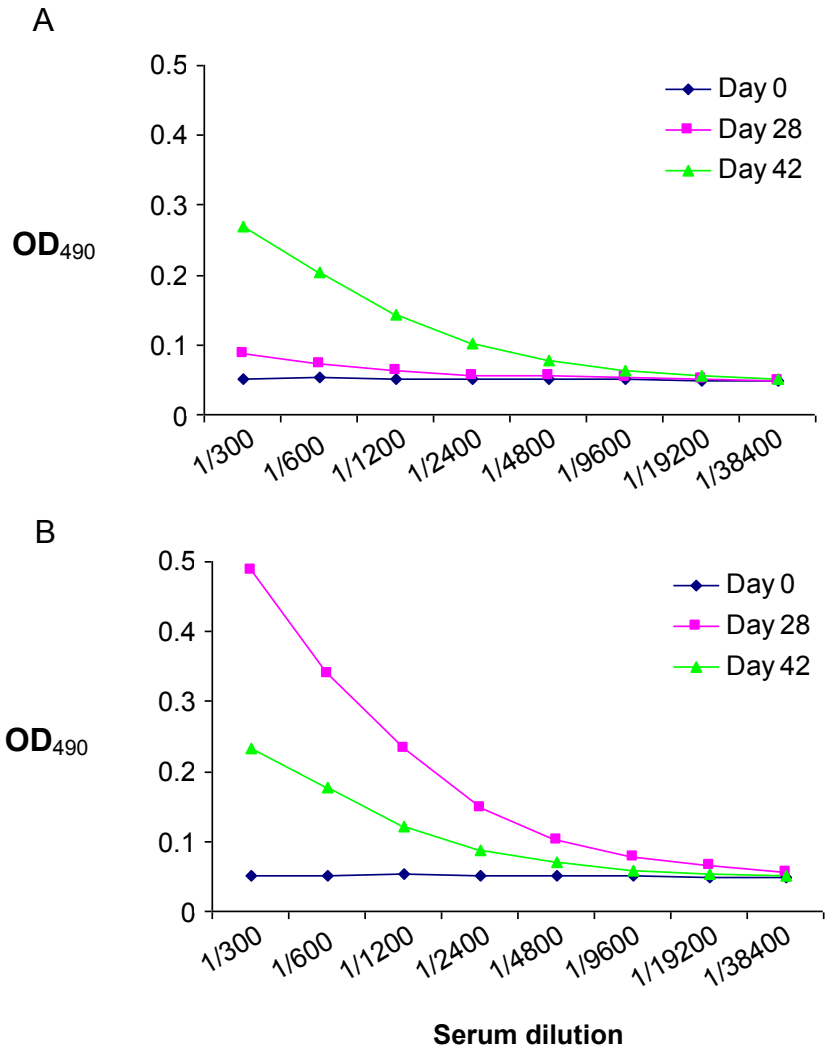


Figure 5.2. Measurement of chTim1-specific serological responses in immunised mice. Immune responses of (A) mouse 1 and (B) mouse 2 were analysed by indirect ELISA, measuring the presence of chTim1-specific antibody in immunised sera, bled on day 28 and 42 post-initial immunisation with pre-immunised sera, bled on day 0, as negative controls. Recombinant chTim1-His6 protein was used as antigen pre-coated on ELISA plates. All sera were 2-fold titrated on plates after an original 1:300 dilution. The secondary Ab is HRP-conjugated goat anti-mouse IgG, followed by a colorimetric assay with an OPD substrate.

after two immunisations, mouse 2 produced a strong immune response, whereas mouse 1 had just started to respond to antigen, compared with pre-immunised sera. After a third immunisation, the response of mouse 1 increased further, but the response of mouse 2 dropped from previous levels.

A His6-tagged chTim4S protein was also used as antigen to detect chTim4-specific antibodies in sera with an indirect ELISA (section 5.2.2). As showed in Figure 5.3, all three mice generated high immune responses against chTim4S-His6 protein after the first two immunisations in comparison with the pre-immunised sera. A third boost stimulated even higher responses in all mice than the previous immunisations.

The chTim4L-hinge-Ig fusion protein was used as antigen in a capture ELISA assay to detect serological immune responses post-immunisation (section 2.6.1). As shown in Figure 5.4A, mouse 1 produced a very strong response after two immunisations, and a third immunisation also increased the response to a higher level. Mouse 2 (Figure 5.4C) had no immune response to antigen after the primary and first booster immunisation, although a third immunisation increased the response. It is therefore difficult to decide if the immune response was fully established. However, ELISA also indicated that all sera positive to the test antigen also had very strong reactive signals to irrelevant Ig fusion protein (rchCD86-Ig) on control plates (Figure 5.4B and D), resulting in no obvious differences of serological reactions between the test and control plates.

To determine specific immune responses against the hinge part of the chTim4L-hinge-Ig fusion protein, and to rule-out responses to the Ig domain, the sera

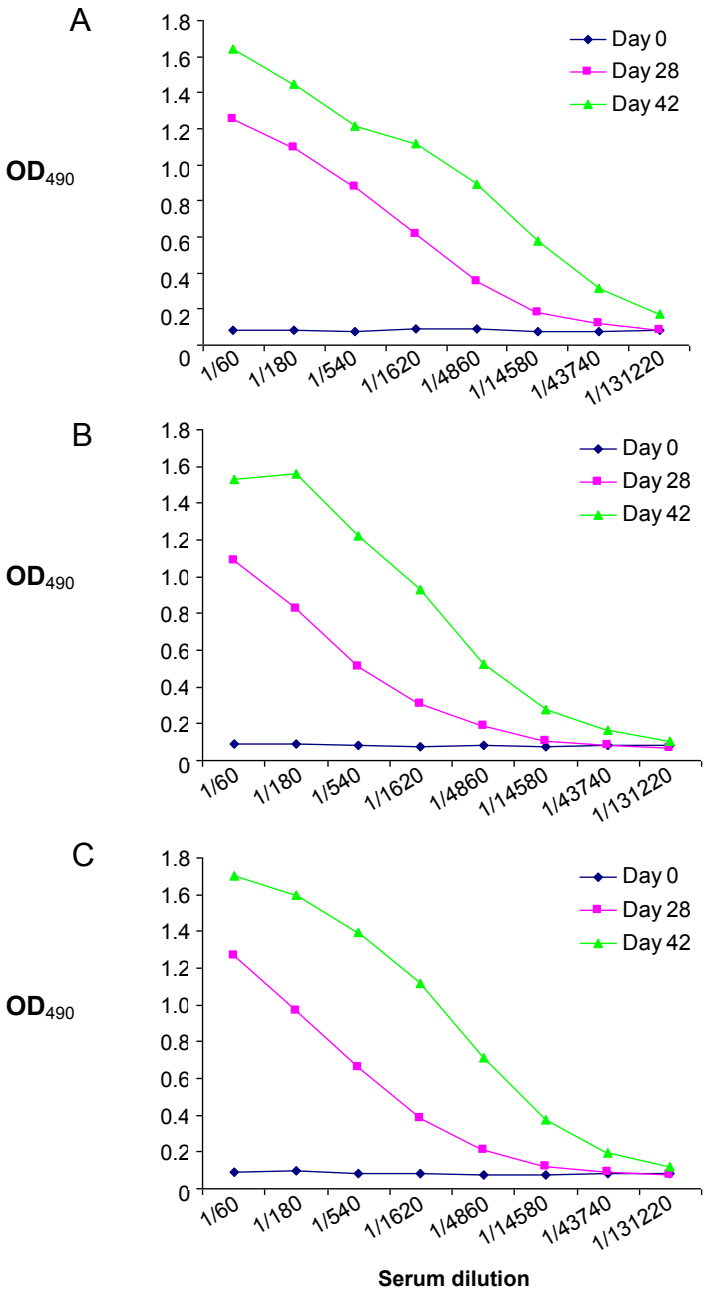


Figure 5.3. Measurement of chTim4S-specific serological immune responses in immunised mice. The responses of (A) mouse 1, (B) mouse 2 and (C) mouse 3 in immunised sera, bled on day 28 and day 42 post-initial immunisation with pre-immunised sera bled on day 0, as negative control, were measured by indirect ELISA assays with chTim4S-His6 protein pre-coated on plates. All sera were 3-fold titrated on plates after an original 1:60 dilution. The secondary Ab is HRP-conjugated goat anti-mouse IgG. The HRP activity was then detected with an OPD substrate.

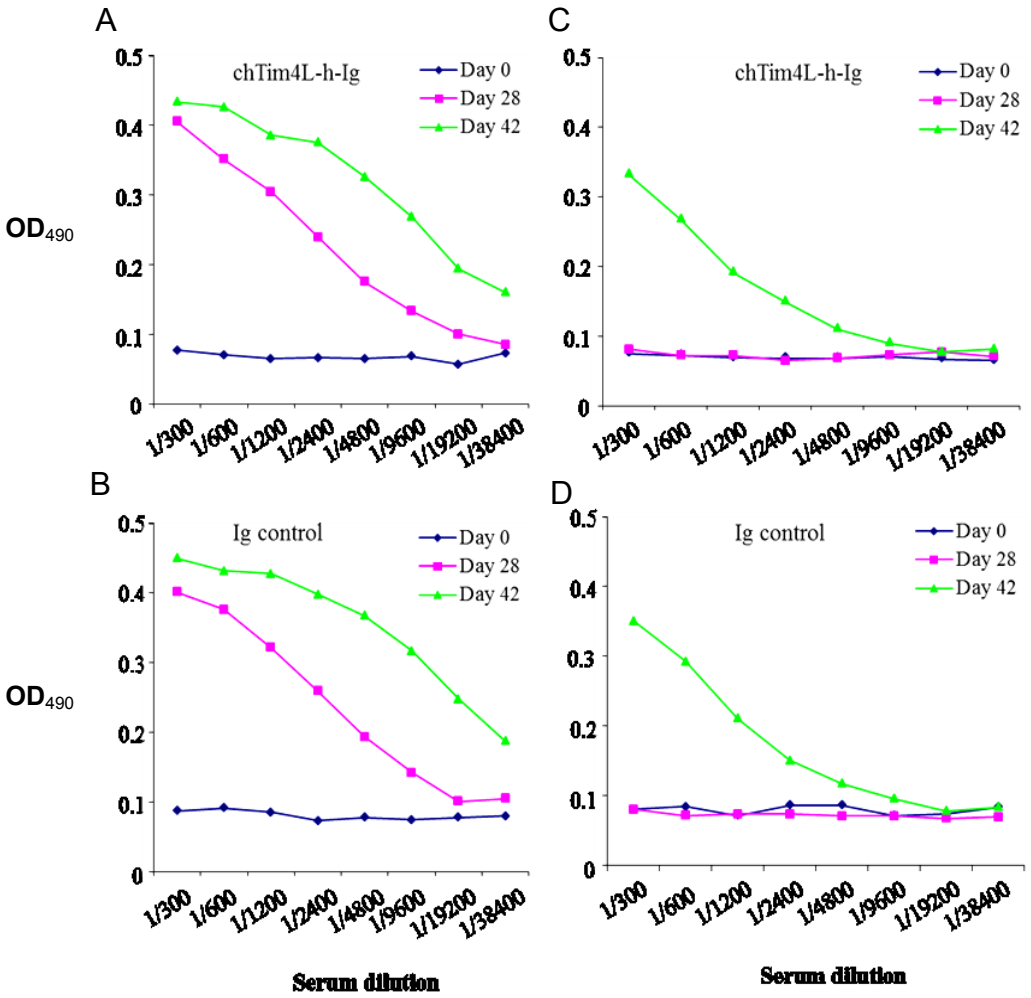


Figure 5.4. Capture ELISA analysis of serological responses against chTim4L-hinge-Ig fusion protein in immunised mice. Recombinant chTim4L-hinge-Ig fusion protein (A, C) and irrelevant Ig fusion protein (rchCD86-Ig) as negative antigen controls (B, D) were captured by pre-coated goat anti-human IgG antibody onto ELISA plates. The responses of mouse 1 (A, B) and mouse 2 (C, D) were analysed by measuring the presence of antibody in immunised sera, bled on day 28 and 42 post-primary immunisation, with pre-immunised sera, bled on day 0, as negative serum controls. All sera were 2-fold titrated on plates after an original 1:300 dilution. The secondary Ab is HRP-conjugated goat anti-mouse IgG. The HRP activity was detected with an OPD substrate.

were incubated with excessive irrelevant Ig fusion protein (rchCD86-Ig), prior to adding to the plate in a competitive ELISA, as described in section 5.2.2. The sera tested were bled after a third immunisation. As shown in Figure 5.5, sera only still strongly reacted with Ig antigen control on the ELISA plate, whereas pre-blocked sera did not interact with it, suggesting blocking with irrelevant Ig completely abolished serological reaction of the anti-Ig antibody. However, as expected, these pre-blocked sera still strongly and dose-dependently bound to the chTim4L-hinge-Ig fusion protein, suggesting that strong immune responses specifically against the hinge part of the chTim4L fusion protein have been successfully established in immunised mice, especially in mouse 1.

5.3.2 Fusions and positive selections of hybridomas

The serological results showed that mouse 1 in both the chTim1-Ig and chTim4L-hinge-Ig immunisations, and mouse 1 and 2 in the chTim4S-Ig immunisation, had established very good immune responses against the respective antigens. Therefore, the spleens were taken after a fourth immunisation and splenocytes isolated from all of these mice for fusions with the partner cells, SP2/0, as described in section 5.2.3.

A single fusion was carried out to generate anti-chTim4L-hinge-Ig monoclonal antibodies and a total of 768 polyclonal hybridomas (eight 96-well plates) were screened by capture ELISA (section 2.6.1). A resultant 143 positive hybidomas produced antibodies against chTim4L-hinge-Ig fusion protein but also reacted with the irrelevant Ig control protein. To distinguish chTim4L-hinge-specific antibodies from the anti-Ig background, a competitive ELISA assay was also used to

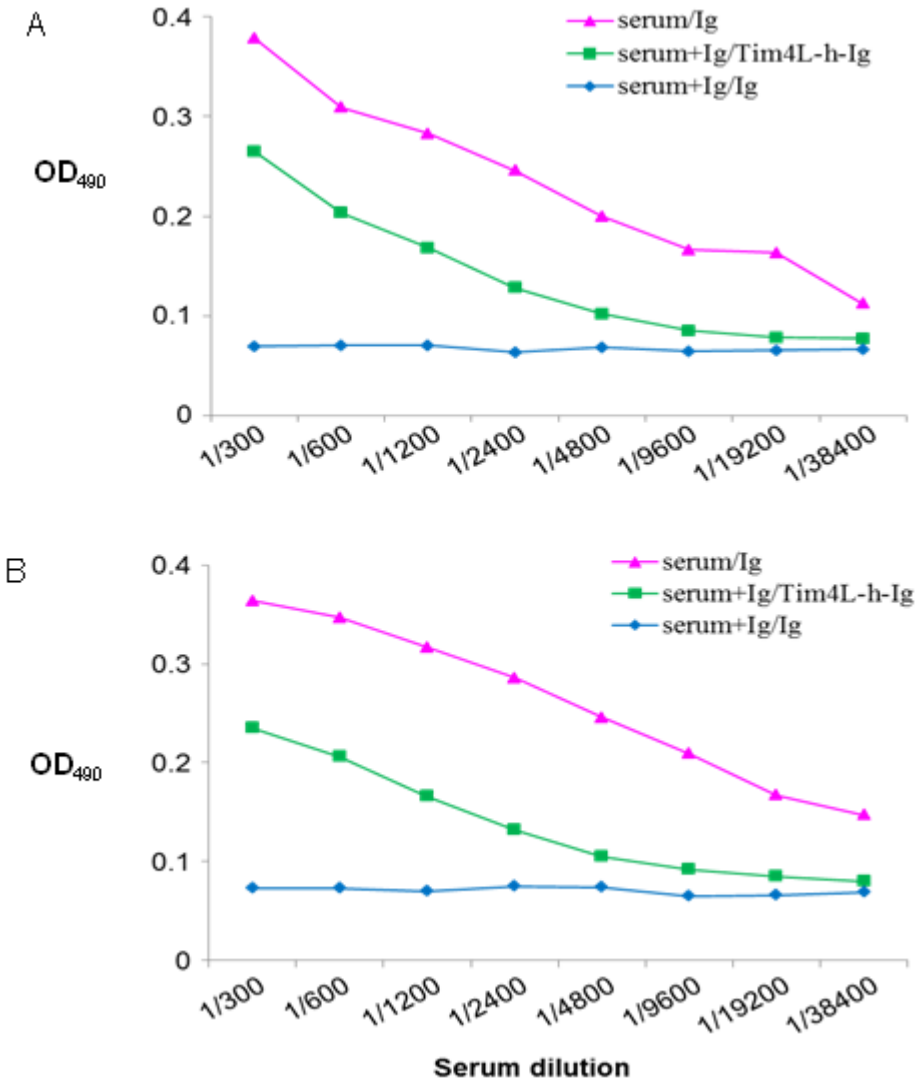


Figure 5.5. Competitive ELISA analysis of specific immune responses against chTim4L-hinge domain. Prior to titration on ELISA plates which pre-coated with chTim4L-hinge-Ig fusion protein and an Ig control antigen (rchCD86-Ig) as control antigen, sera from mice with three immunisations were pre-incubated with or without excessive irrelevant Ig fusion protein (rchCD86-Ig) to remove the detection of anti-Ig Ab. All sera were 2-fold titrated on plates after an original 1:300 dilution. The bound primary Ab was detected by an HRP-conjugated goat anti-mouse IgG Ab, followed by colourisation of OPD substrate. Serum/Ig in pink = untreated sera reaction with immobilised Ig control on the plate; Serum+Ig/Tim4-h-Ig in green = Ig-blocked sera reaction with chTim4L-hinge-Ig; Serum+Ig/Ig in blue = Ig-blocked sera reaction with Ig control. (A) mouse 1 and (B) mouse 2.

re-screen these positive antibodies, in which an irrelevant Ig-fusion protein (rchCD86-Ig) was used to block the supernatants prior to adding to antigens on plates, as described in section 5.2.2. Eventually, 13 hybridomas were identified positively secreting antibodies specifically against the hinge part of the chTim4L fusion protein but not the Ig domain. After two rounds of subcloning of all these polyclonal hybrydomas, five monoclonal hybridomas, as listed in Table 5.1, were eventually obtained to produce specific mAbs against the hinge part of chTim4L.

A single fusion was also carried out to generate anti-chTim1 monoclonal antibodies and a total of 768 polyclonal hybridomas (eight 96-well plates) were screened by indirect ELISA (section 5.2.2). 102 positive hybridomas were identified and then grown up in 24-well plates. 56 hybridomas survived and were expanded. Only 8 of these secreted chTim1-specific antibodies. After two rounds of subcloning of these hybridomas, finally, 3 monoclonal hybridomas, as listed in Table 5.1, were established to sustainably produce anti-chTim1 mAbs.

Two fusions were performed individually with the splenocytes isolated from mouse 1 and 2 respectively immunised with the chTim4S-Ig fusion protein. Approximately 2,000 hybridomas (twenty 96-well plates) were screened by indirect ELISA (section 5.2.2) and 256 positive hybridomas were obtained. They were then expanded on 24-well plates and 154 hybridomas survived, among which 47 produced chTim4S-specific antibodies. These hybridomas were ranked by ELISA analysis and 9 hybridomas were chosen to perform subcloning. Eventually, 5 monoclonal hybridomas, as listed in Table 5.1, were obtained after two rounds of subcloning.

Antigen	Clone	Subclass
chTim1	EG7	IgG2a
	CF3	IgG2a
	GG9	IgG2a
chTim4S	JH9	IgG1
	BC8	IgG1
	JF1	IgG1
	AA12	IgG1
	FA1	IgG1
chTim4L-hinge	EA3	IgG1
	AE7	IgG1
	BE6	IgG2b
	IE12	IgG2a
	DD9	IgG2a

Table 5.1. List of mAbs against chTim molecules.

5.3.3 Anti-chTim1 antibody specificity and epitopes recognised, as determined by ELISA analysis

The established monoclonal hybridomas were cultured in large scale and the supernatants harvested. The mAbs were then purified with protein G columns,as described in section 2.9. The purified mAbs were analysed for their antigen specificity by ELISA.

To characterise the specificity of the anti-chTim1 mAbs, they were titrated on chTim1-His6-coated ELISA plates, along with anti-chTim4L-hinge and isotype control mAbs. The bound antibodies were measured by colorimetric assay, as shown in Figure 5.6. Anti-chTim1 mAbs specifically recognise the chTim1-His6 protein, as

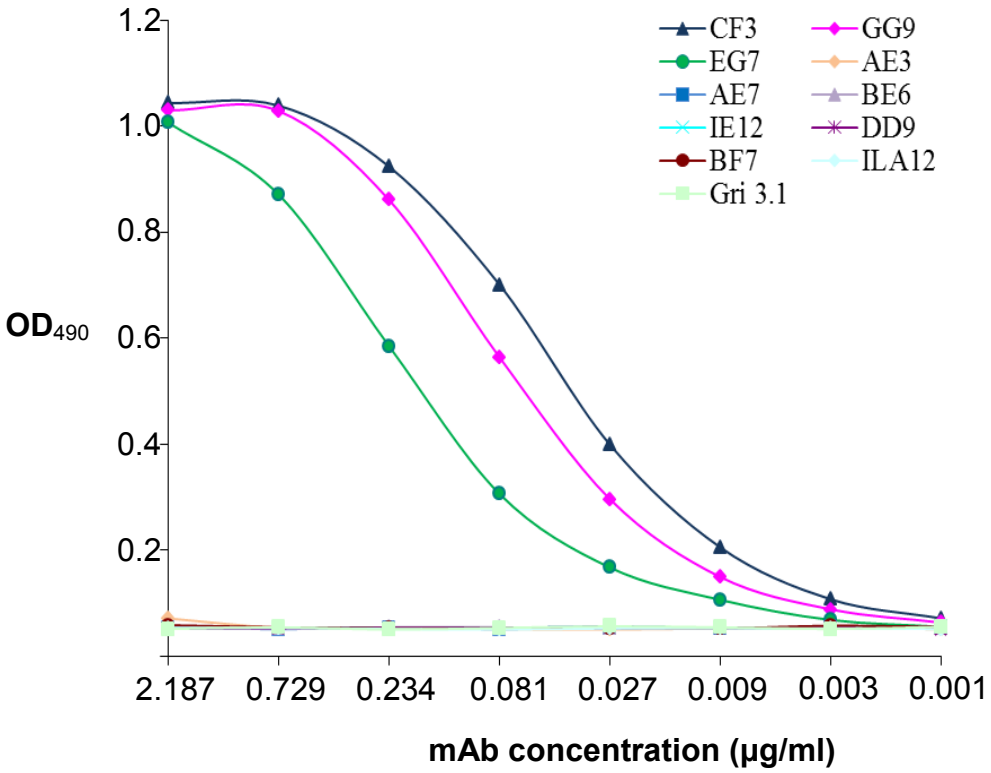


Figure 5.6. Indirect ELISA analysis of specificity of anti-chTim1 mAbs. ChTim1-His6 protein was used as antigen pre-coated on ELISA plates. Anti-chTim1 mAbs (CF3, GG9 and EG7), anti-chTim4L-hinge mAbs (AE3, AE7, BE6, IE12 and DD9) and isotype control mAbs, ILA12 (IgG2a) and Gri 3.1(IgG1) were titrated on the plates. A HRP-conjugated goat anti-mouse-IgG Ab was used to detect bound primary antibody. OPD substrate was used to detect the HRP activity.

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compared to isotype control mAbs. As expected, anti-chTim4L-hinge mAbs did not cross-react with the chTim1-His6 antigen, only producing background interactions, similar to those of the isotype mAb controls.

To further determine the binding sites of the anti-chTim1 mAbs, a capture ELISA (section 2.6.1) was used to determine if they bound to different domains of the chTim1 protein, including the chTim1-IgV-Ig and chTim1-mucin-Ig fusion proteins as targets, as shown in Figure 5.7. The three anti-chTim1 mAbs all dose-dependently bound to the mucin domain of the chTim1 molecule. By contrast, only background binding to the IgV domain of chTim1 was seen, suggesting these anti-chTim1 mAbs recognise epitopes mapping to the mucin domain of chTim1 but not to the IgV domain.

To determine if the epitopes recognised by the three anti-chTim1 mAbs were closely located, a competitive ELISA (section 5.2.7) was used. Unlabelled anti-chTim1 mAbs were used to block the binding sites on chTim1-His6 pre-coated on ELISA plates, prior to the addition of biotinylated anti-chTim1 mAbs. The bound biotinylated mAbs were detected by HRP-conjugated streptavidin, as shown in Figure 5.8. The pre-incubation of any of the anti-chTim1 mAbs dramatically reduced the binding of the other two anti-chTim1 mAbs to the chTim1 antigen, suggesting that the epitopes of the three anti-chTim1 mAbs may be closely associated. However, the binding efficiency of the three anti-Tim1 mAbs to their antigen shows variation (Figures 5.6 and 5.7), with CF3 high, GG9 intermediate and EG7 relatively low.

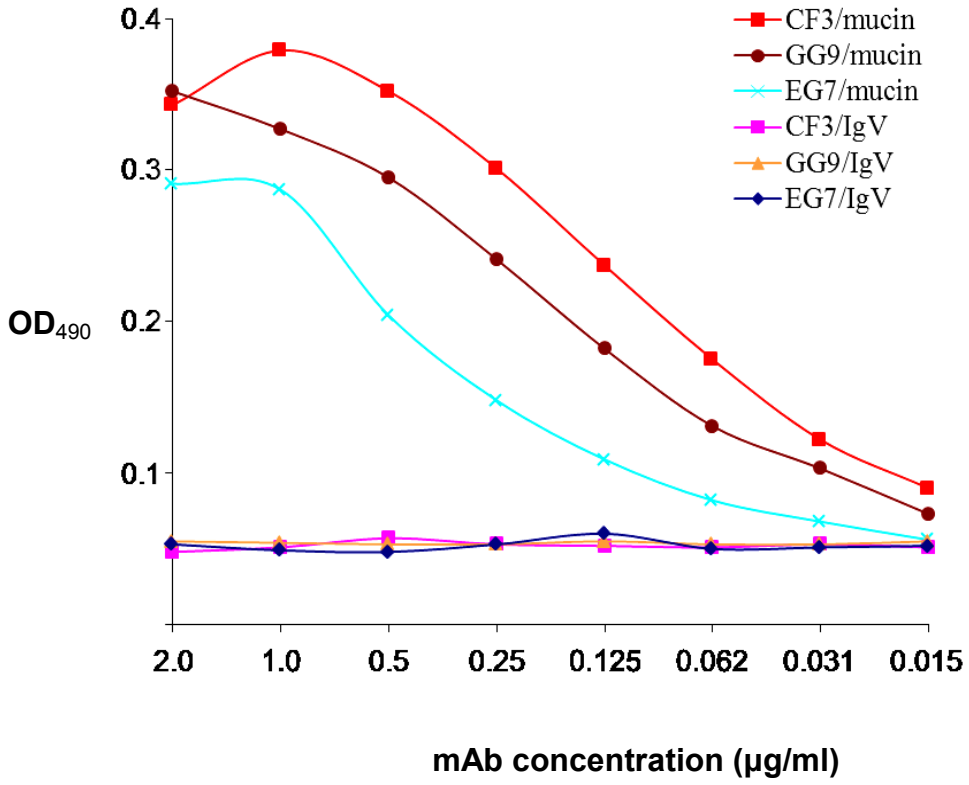


Figure 5.7. Epitope mapping of anti-chTim1 mAbs, as detected by a capture ELISA. Recombinant chTim1-IgV-Ig (IgV) and chTim1-mucin-Ig (mucin) fusion proteins, as antigens, were captured onto ELISA plates by pre-coated goat anti-human IgG antibody, and anti-chTim1 mAbs, including CF3, GG9 and EG7, were titrated on this plate. An HRP-conjugated goat anti-mouse IgG antibody was used to detect bound primary antibodies. OPD substrate was used to detect HRP bioactivity.

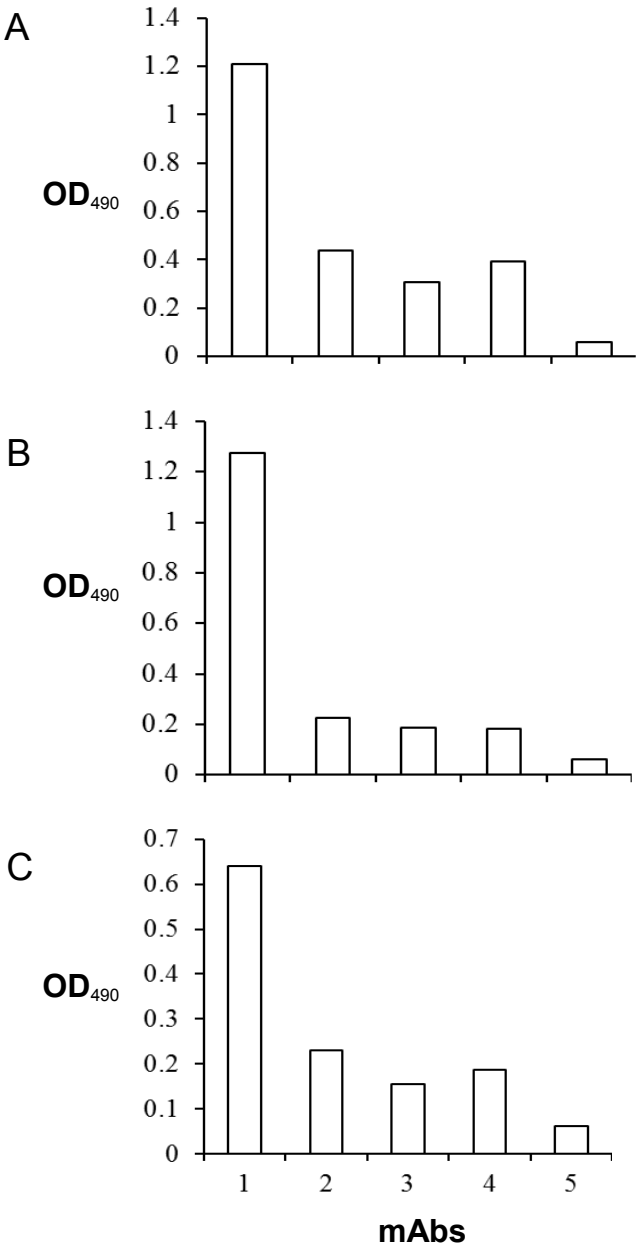


Figure 5.8. Analysis of the epitopes bound by the anti-Tim1 mAbs by a competitive ELISA. ChTim1-His6, as an antigen pre-coated on the plate, was incubated with unlabelled anti-chTim1 mAbs, EG7 (2), CF3 (3), GG9 (4) and PBS (1, 5). After washing the plate, biotinylated anti-chTim1 mAbs were added to 1, 2, 3 and 4, and a biotinylated control mAb (ILA12) to 5, to compete for binding to the antigen. Bound biotinylated Abs were detected by HRP-conjugated streptavidin, followed by colorimetric assay with an OPD substrate. (A) biotinylated CF3, (B) biotinylated GG9 and (C) biotinylated EG7.

5.3.4 Anti-chTim4 antibody specificity and epitopes recognised, as detected by ELISA analysis

To characterise the specificity of the anti-chTim4S mAbs, an indirect ELISA (section 5.2.2) was used with chTim4S-His6 protein as the target antigen, and chTim1-His6 protein as a negative control, as shown in Figure 5.9. Anti-chTim4S mAbs specifically recognised chTim4S-His6 protein, but did not cross-react with the control antigen.

To determine the location of the epitopes recognised by the anti-chTim4S mAbs, a capture ELISA (section 2.6.1) was used with chTim4S-IgV-Ig and chTim4S-mucin-Ig fusion proteins as antigens, along with an irrelevant Ig negative control, rchCD86-Ig fusion protein, as shown in Figure 5.10A. Four anti-chTim4S mAbs, including FA1, JH9, JF1 and BC8, strongly and dose-dependently bound to the chTim4S-IgV-Ig fusion protein compared to their binding to the negative Ig control; by contrast, these mAbs did not bind to the chTim4S-mucin-Ig protein, suggesting the epitopes recognised by them are on the IgV domain of chTim4S. One anti-chTim4S mAb, AA12, had only background binding to either antigen when compared with the negative Ig control (Figure 5.10A), suggesting its epitope is neither on the IgV nor the mucin domain of chTim4S. However, AA12 was previously shown to bind to the chTim4S-His6 protein (Figure 5.9), suggesting AA12 recognises an epitope comprising elements of both the IgV and mucin domains of chTim4S. To confirm this prediction, a capture ELISA was used to re-map the epitope recognised by AA12, where IgV, mucin and whole extracellular (IgV+mucin) domain chTim4S fusion proteins were captured by their fused-Ig tag, along with an irrelevant Ig negative control, rchCD86-Ig, as shown in Figure 5.10B.

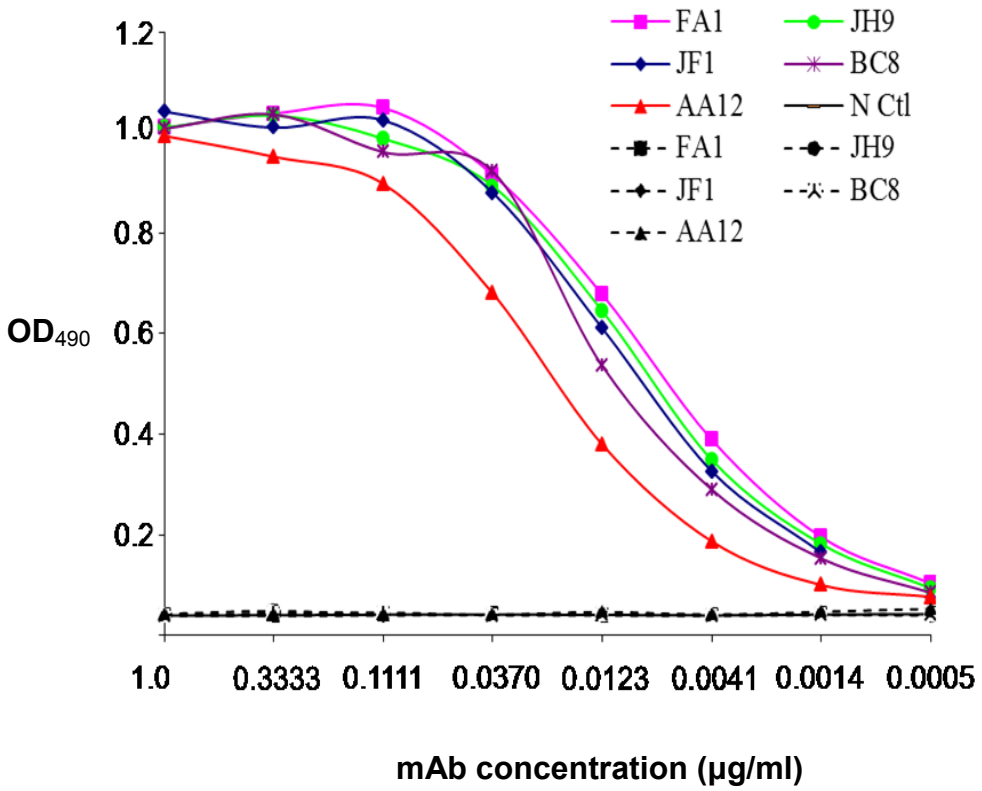


Figure 5.9. Specificity of anti-chTim4S mAbs analysed by an indirect ELISA. Recombinant chTim4S-His6 and chTim1-His6 proteins, as antigens, were coated onto ELISA plates. Anti-chTim4S mAbs, including FA1, JH9, JF1, BC8 and AA12, were then 3-fold titrated on this plate. Bound primary mAbs were detected by an HRP-conjugated goat anti-mouse IgG antibody, followed by colorimetric assay with an OPD substrate. N Ctl = negative controls, wells containing no coating antigen, nor adding a primary antibody. Solid lines represent interactions of the antibodies with chTim4S, and dashed black lines interactions with chTim1.

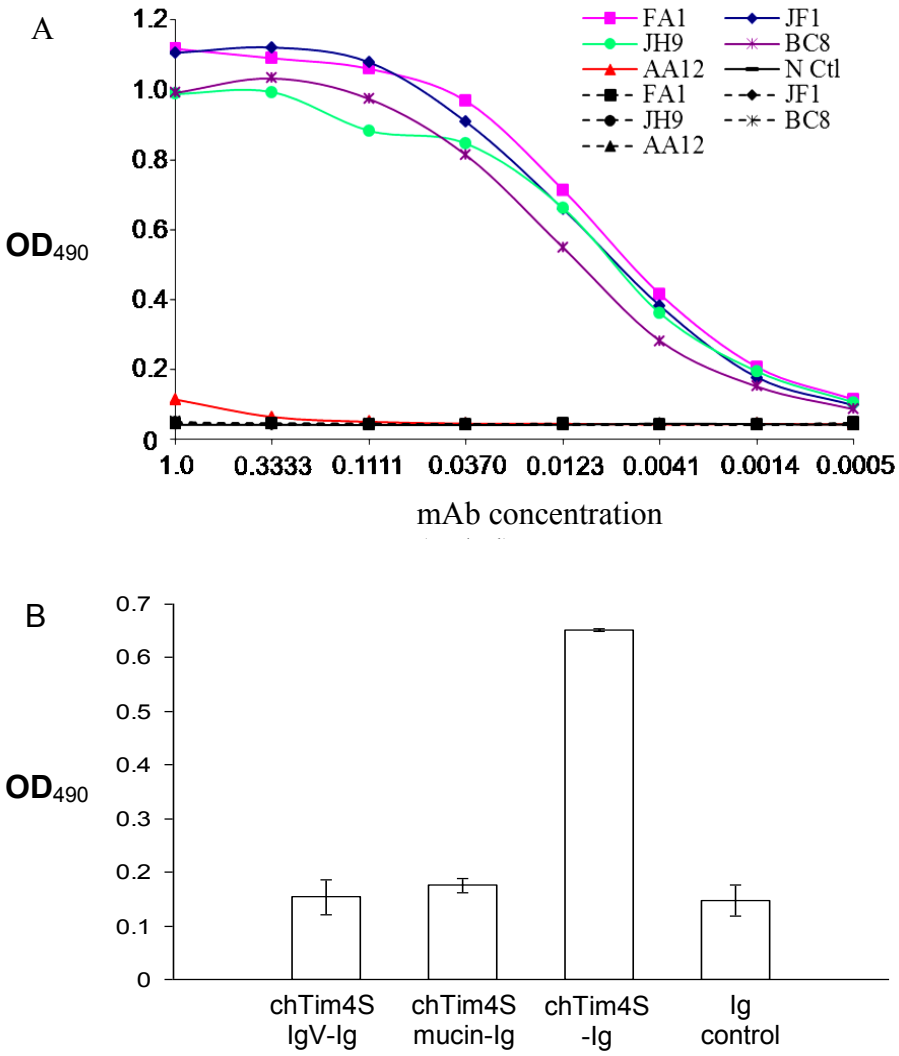


Figure 5.10. Epitopes of anti-chTim4S mAbs analysed by capture ELISA. (A). ChTim4S-IgV-Ig and chTim4S-mucin-Ig fusion proteins were captured on ELISA plates pre-coated with goat anti-human IgG antibody, and anti-chTim4S mAbs, including FA1, JF1, JH9, BC8 and AA12, were then 3-fold titrated on the plate. Solid colour lines represent interaction of the mAbs with the chTim4S-IgV-Ig protein and dotted black lines are interaction with the chTim4S-mucin-Ig protein. N Ctl = negative control, where wells are neither coated with antigen, nor a primary antibody added. (B). ChTim4S-IgV-, -mucin- and -extracellular-Ig fusion proteins and an irrelevant Ig (rchCD86-Ig) as negative control, were captured by pre-coated goat anti-human IgG antibody in triplicate wells on plate, followed by the addition of the mAb AA12. Bound AA12 was detected by an HRP-conjugated goat anti-mouse IgG antibody. Error bars show the standard error from three repeated wells.

Consistently, AA12 specifically bound to the whole extracellular domain chTim4S fusion protein in comparison with the negative Ig control, but not to the IgV or mucin domain chTim4S fusion proteins.

To characterise the specificity of the anti-chTim4L-hinge mAbs, chTim4L-hinge-Ig fusion protein was used as antigen in a capture ELISA (section 2.6.1), which also included isotype control mAbs, ILA12 and GRi3.1, as shown in Figure 5.11. The anti-chTim4L-hinge mAbs specifically recognised chTim4L-hinge-Ig fusion protein when compared with the isotype control mAbs. However, the binding efficiency of the antibodies for the antigen appeared to be variable. Three anti-chTim4L-hinge mAbs, including EA3, AE7 and IE12, bound with high amount, but DD9 and BE6 appeared to be low and very low, respectively.

To further characterise the ability of the mAbs to recognise full-length chTim4L protein, plasmid encoding full-length chTim4L cDNA was transfected into COS-7 cells (section 2.7.2), followed by immunostaining with anti-chTim4L-hinge mAbs, including EA3, AE7, IE12 and DD9 (section 2.8). The staining results were analysed by confocal microscopy, as shown in Figure 5.12. Antibodies EA3, IE12 and DD9 positively stained some, but not all of the transfected COS-7 cells in the same observed field, suggesting these antibodies stain the positively transfected COS-7 cells expressing chTim4L protein. However, the staining was mostly concentrated around nuclei, suggesting that the expressed chTim4L proteins are located in that area, an observation consistent with the expression of chTim4L soluble protein, which was previously shown to be intracellularly expressed in the ER in transfected COS-7 cells (Figures 3.14 and 3.16). Surprisingly, mAb AE7 apparently stained the nucleus of most transfected COS-7 cells, suggesting it may

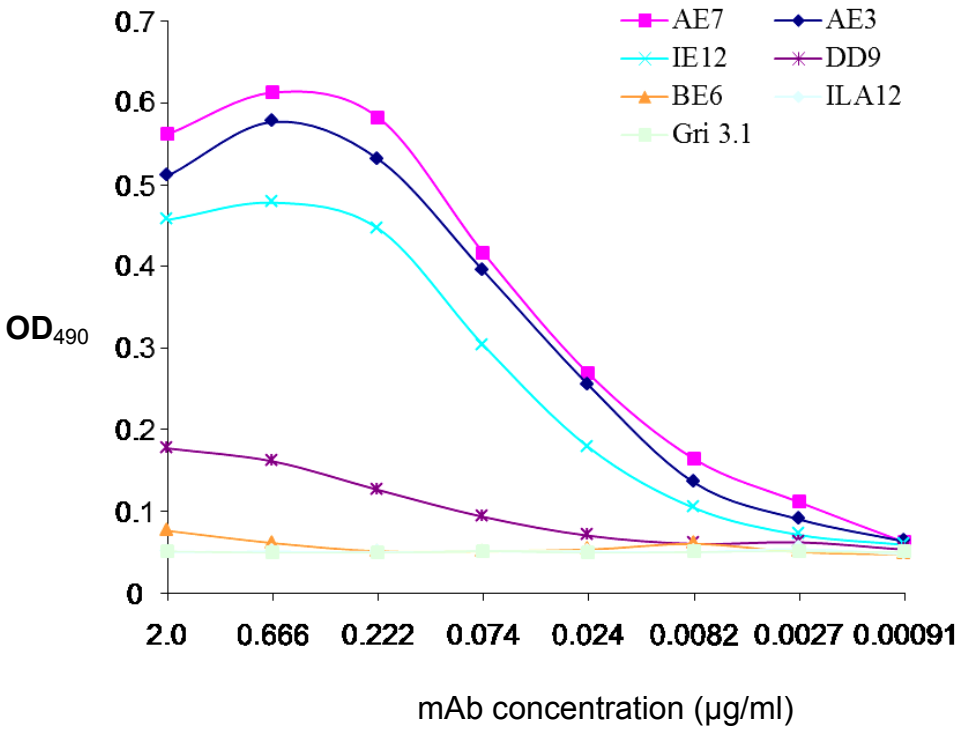


Figure 5.11. Specificity of anti-chTim4L-hinge mAbs analysed by an indirect ELISA. ChTim4L-hinge-Ig fusion protein was used as an antigen pre-coated on ELISA plates. The anti-chTim4L-hinge mAbs, including AE3, AE7, BE6, IE12 and DD9, and isotype control mAbs, ILA12 (IgG2a) and Gri 3.1 (IgG1), were then 3-fold titrated on the plates. Bound primary mAbs were detected by an HRP-conjugated goat anti-mouse IgG antibody. OPD substrate was used to detect the HRP activity.

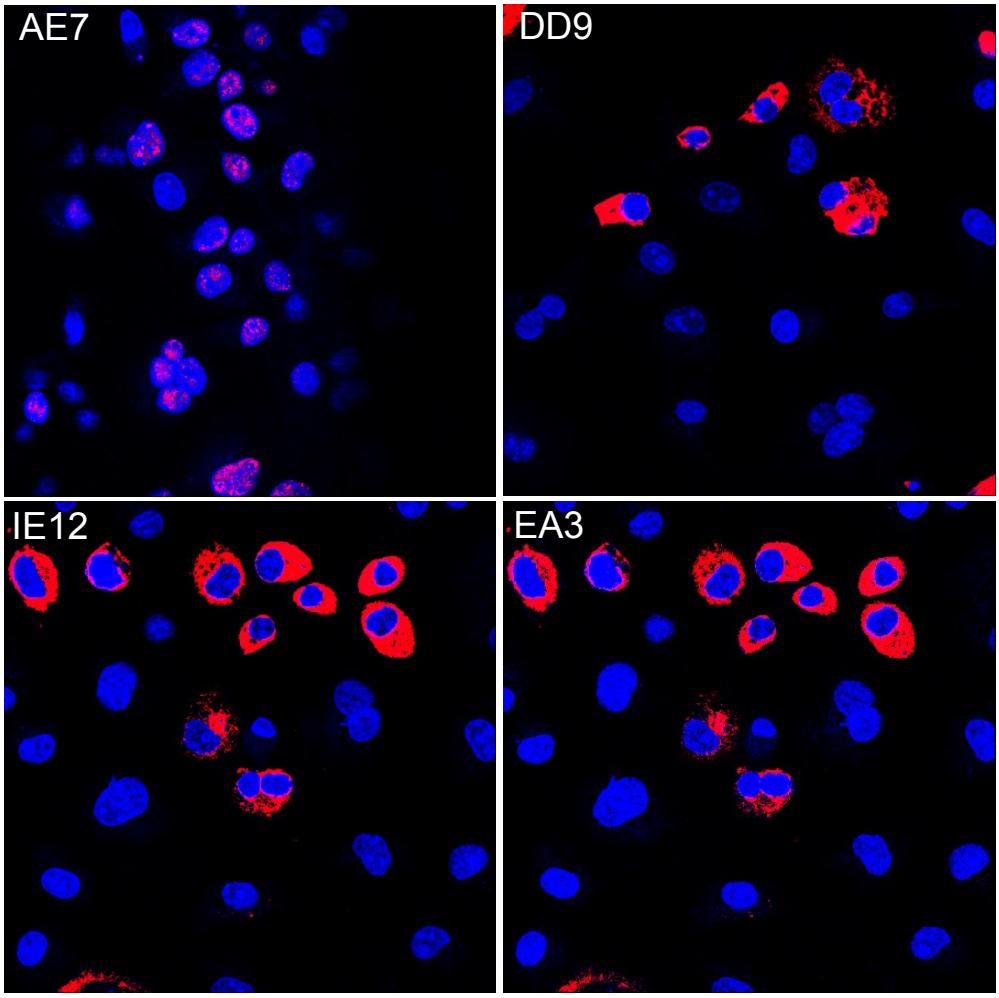


Figure 5.12. Confocal microscopy of anti-chTim4L-hinge mAbs immunostaining transfected COS-7 cells. Vectors expressing full-length chTim4L cDNAs were transiently transfected into COS-7 cells, followed by immunostaining the cells with the anti-chTim4L-hinge mAbs, AE7, DD9, IE12 and EA3. Bound primary mAbs were detected by Alex Fluor 586-conjugated goat anti-mouse IgG antibody in red colour, and nuclei were counter-stained with DAPI in blue.

cross-react with an unknown nuclear antigen in those cells.

5.3.5 Specificity of anti-chTim mAbs characterised with transiently transfected COS-7 cells

To determine if the anti-chTim mAbs recognise their cognate antigen expressed in transfected cells, the full-length respective chTim cDNAs in expression vectors were transiently transfected into COS-7 cells (section 2.7.2). The transfected COS-7 cells were fixed by paraformaldehyde and then immunostained with the anti-chTim mAbs (section 2.8). Consistent with the previous ELISA results, the anti-chTim1 mAbs (Figure 5.13), including CF3, EG7 and GG9, specifically recognised chTim1-transfected COS-7 cells in comparison with the control isotype mAb (ILA12) staining. They did not cross-react with chTim4S- and chTim4L-transfected COS-7 cells.

Although the anti-chTim4L-hinge mAbs (Figure 5.14), including AE7, IE12 and EA3, specifically recognised the chTim4L antigen expressed by transfected COS-7 cells in comparison with the isotype control mAbs GRi3.1 and ILA12, the strength of interaction between antibody and antigen was variable. IE12 and EA3 strongly bound to chTim4L-transfected cells, but EA3 bound slightly less than IE12. Similar to the previous confocal results (Figure 5.12), AE7 mAb bound to transfected cells, but the binding seemed to be condensed to nuclei. DD9 had very low or undetectable staining of chTim4L-transfected COS-7 cells. These results are consistent with the previous observations with ELISA (Figure 5.11) and confocal analysis (Figure 5.12). As expected, the anti-chTim4L-hinge mAbs did not cross-react with either chTim4S- or chTim1-transfected COS-7 cells.

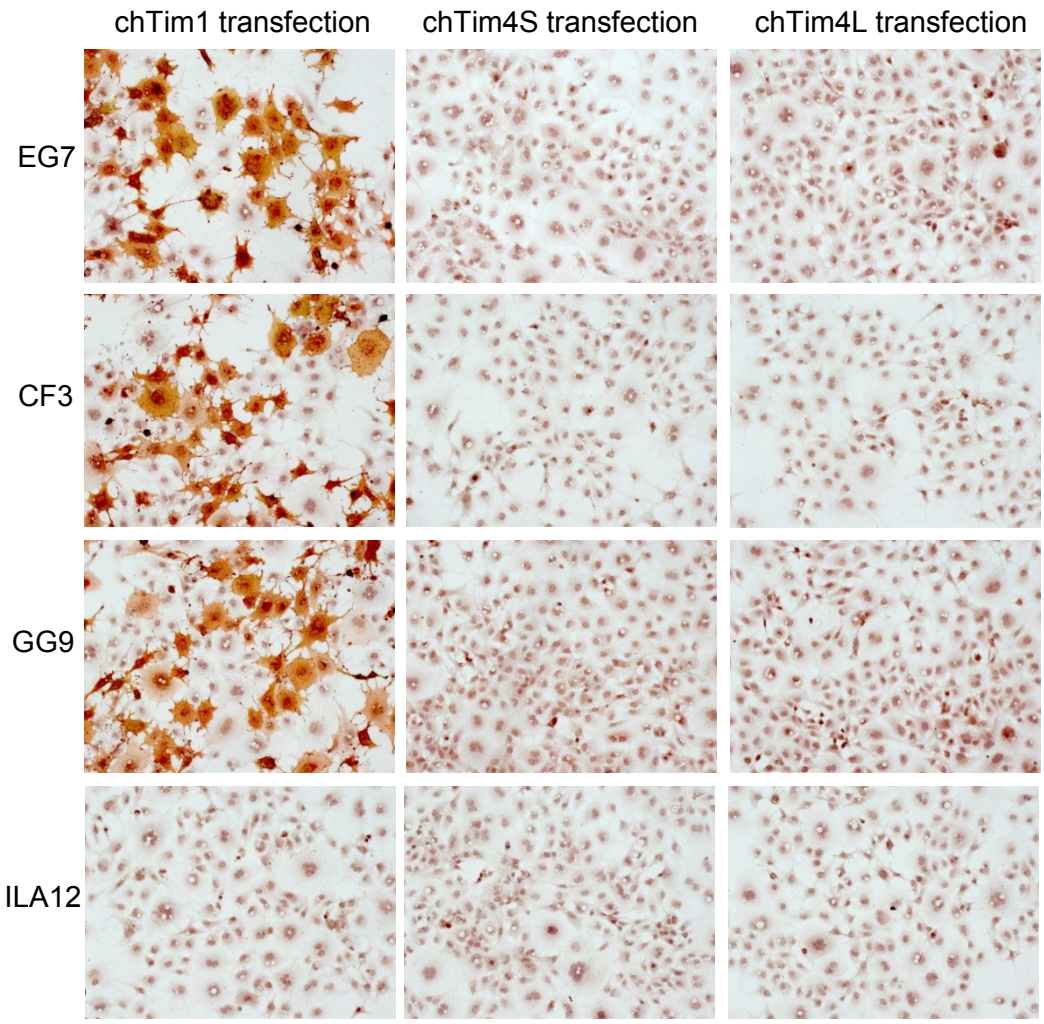


Figure 5.13. Immunostaining of COS-7 transfectants with anti-chTim1 mAbs. Vectors expressing full-length cDNAs of chTim1 (left panel), chTim4S (middle panel) or chTim4L (right panel) were transiently transefected into COS-7 cells, followed by immnostaining the cells with anti-chTim1 (EG7, CF3 and GG9) and isotype controls ILA12 (IgG2a) mAbs. The secondary antibody was biotinylated goat anti-mouse IgG, followed by HRP-labelled straptavidin. AEC chromogen was used to detect HRP bioactivity, shown as red. The nuclei were counter-stained by hematoxylin, shown as purple. The positive staining showed cells were in dark red when compared to negative staining.

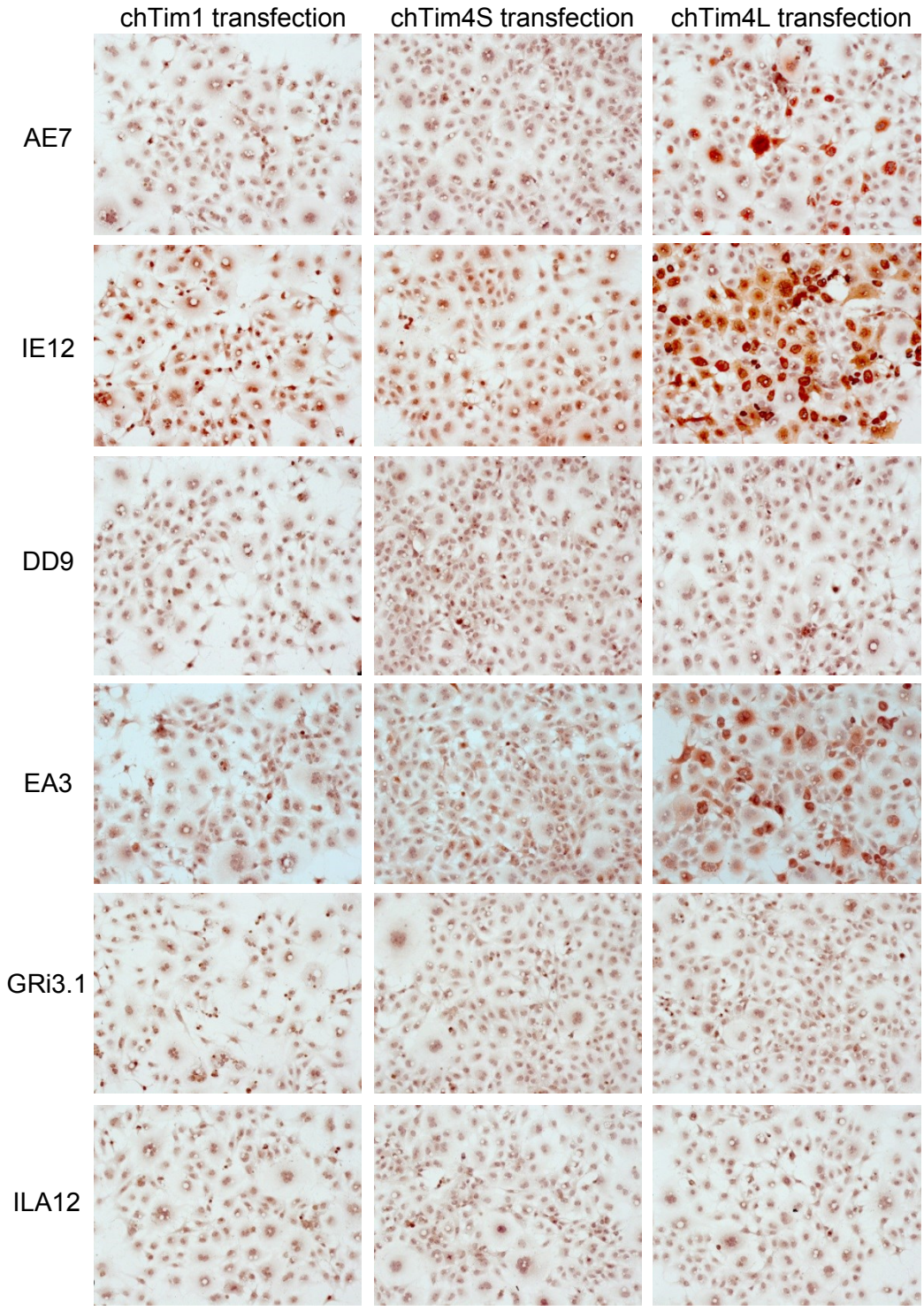

chTim1 transfection
chTim4S transfection
chTim4L transfection
AE7
IE12
DD9
EA3
GRi3.1
ILA12

Figure 5.14. Immunostaining of COS-7 transfectants with anti-chTim4L-hinge mAbs. Vectors expressing full-length cDNAs of chTim1 (left panel), chTim4S (middle panel) or chTim4L (right panel) were transiently transefected into COS-7 cells, followed by immnostaining the cells with anti-chTim4L-hinge (EA3, AE7, IE12 and DD9) mAbs. GRi3.1 (IgG1) and ILA12 (IgG2a) are isotype controls. The secondary antibody was biotinylated goat anti-mouse IgG, followed by HRP-labelled straptavidin. AEC chromogen was used to detect HRP bioactivity, shown as red. The nuclei were counter-stained by hematoxylin, shown as purple. The positive staining showed cells were in dark red when compared with negative staining.

As predicted, the anti-chTim4S mAbs (Figure 5.15), AA12, BC8, JF1, JH9 and FA1, recognised not only chTim4S, which is consistent to previous results by ELISA analysis (Figure 5.9), but also chTim4L antigens expressed by transfected COS-7 cells, when compared with the isotype control mAb, GRi3.1. However, all five antibodies did not cross-react with chTim1- transfected cells. One anti-chTim4S mAb, JF1, appeared to have a different staining pattern on chTim4S- and chTim4L-transfected COS-7 cells, strongly binding to the former but less strongly to the latter (Figure 5.15).

5.3.6 Characterisation of anti-chTim mAbs by western blot analysis

To further characterise the anti-Tim4S mAbs and determine molecular weights of the full-length chTim4S and chTim4L proteins, cell lysates, obtained from previously transfected COS-7 cells (section 5.2.8), were used for western blot analysis, as shown in Figure 5.16. MAbs FA1 and JF1 strongly bound to bands at approximately 33 kD in chTim4S- and chTim4L-transfected cell lysates, but very weakly to a similar size band in chTim1-transfected cell lysate. JH9 and AA12 mAbs can detect not only 33 kDa bands, but also a 75 kDa band in chTim4S-transfected

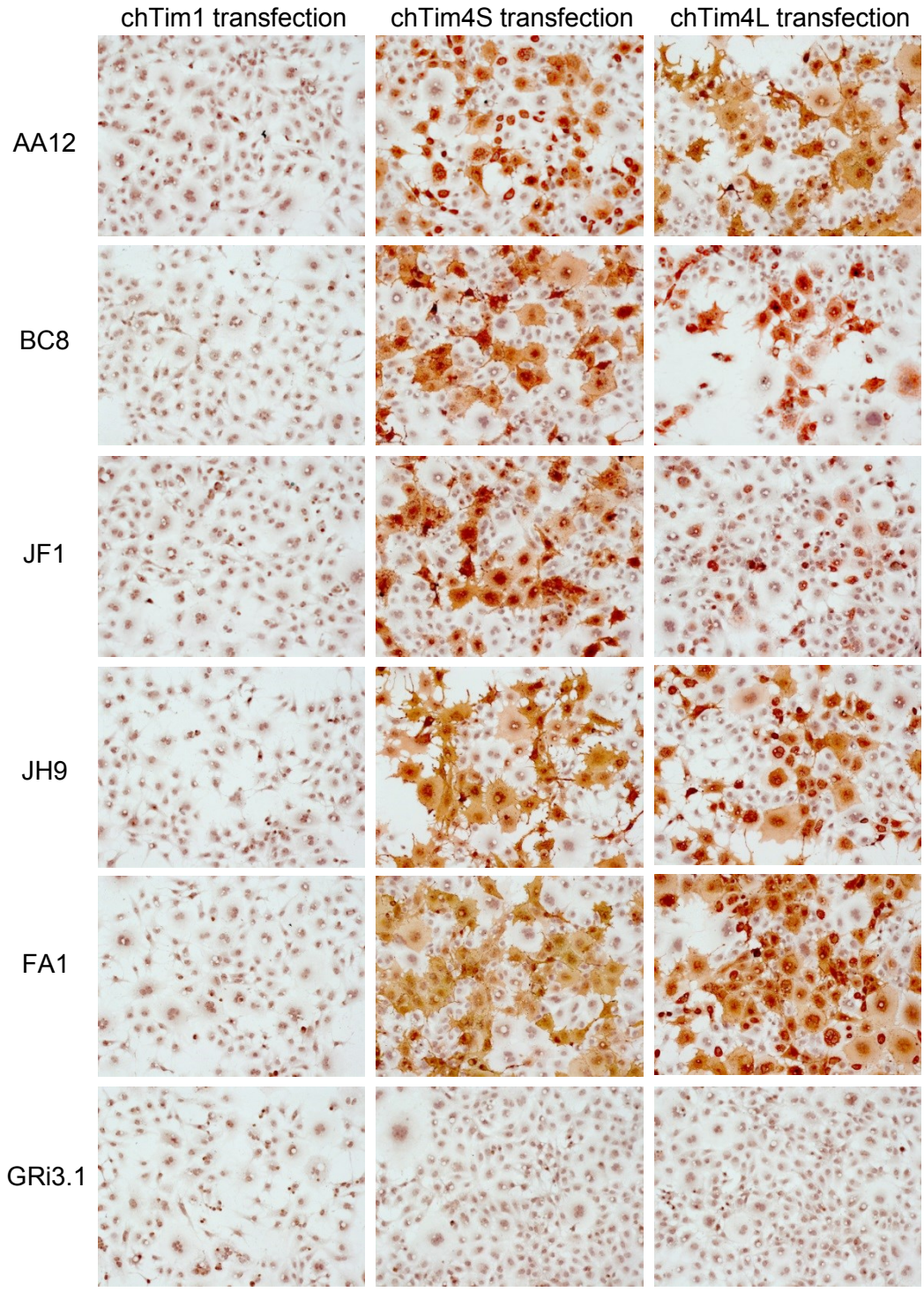

chTim1 transfection
chTim4S transfection
chTim4L transfection
AA12
BC8
JF1
JH9
FA1
GRi3.1

Figure 5.15. Immunostaining of COS-7 transfectants with anti-chTim4S mAbs. Vectors expressing full-length cDNAs of chTim1 (left panel), chTim4S (middle panel) or chTim4L (right panel) were transiently transefected into COS-7 cells, followed by immnostaining the cells with anti-chTim4S (AA12, BC8, JF1, JH9 and FA1) and isotype controls Gri3.1 (IgG1) mAbs. The secondary antibody was biotinylated goat anti-mouse IgG, followed by HRP-labelled straptavidin. AEC chromogen was used to detect HRP bioactivity, shown as red. The nuclei were counter-stained by hematoxylin, shown as purple. The positive staining showed cells were in dark red when compared to negative staining.

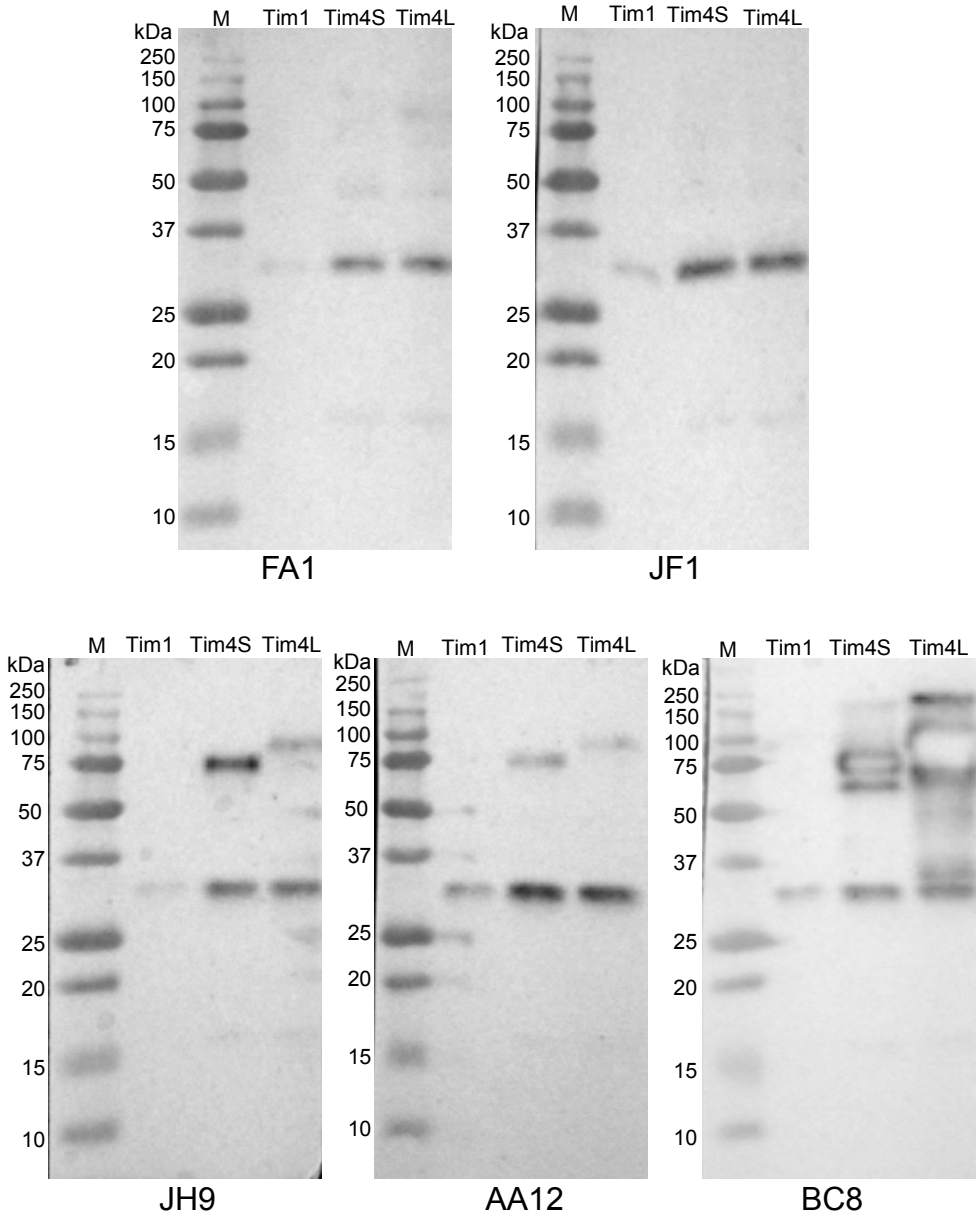


Figure 5.16. Characterisation of chTim4S mAbs by western blot. Transfected COS-7 cell lysates, containing full-length chTim proteins, were used for western blots. The anti-chTim4S mAbs, as labelled beneath each picture, were used to probe the blots, followed by an HRP-conjugated goat anti-mouse IgG Ab for detection of bound primary mAbs. ECL reagent was used to detect HRP bioactivity. Tim1 = Tim1-transfected COS-7 cell lysate, Tim4S = Tim4S-transfected COS-7 cell lysate, Tim4L = Tim4L-transfected COS-7 cell lysate, M = Precision Plus protein standard (all blue, BioRad).

cell lysate and a 95 kDa band in the chTim4L-transfected cell lysate. BC8 detected even more bands, 75, 70 and 33 kDa bands in cell lysate containing chTim4S, and 250, 95 and 33 kDa bands in cell lysate containing chTim4L, but bound most strongly to the 75 kDa and 95 kDa bands. The major 75 and 95 kDa bands in the respective chTim4S- and chTim4L-transfected cell lysates are bigger than the molecular mass of 36.9 kDa of full length chTim4S and 57.3 kDa of full-length chTim4L, as predicted using the Protparam programme (http://web.expasy. org/cgi-bin/protparam/protparam). This suggests that chTim4S and chTim4L protein may be highly glycosylated, and they do possess multiple N- and O-linked glycosylation sites. However, it is not clear why all the anti-chTim4S mAbs recognised a 33 kDa band in the chTim4S- and chTim4L-transfected cell lysates.

The expression of ChTim1 and chTim4L by transfected COS-7 cells was also determined by western blot, where GG9 (anti-chTim1) and IE12 (anti-chTim4L-hinge) mAbs were used to detect the presence of expressed proteins, as shown in Figure 5.17. IE12 specifically bound to a band of 95 kDa in the chTim4L-transfected COS-7 cell lysate and did not cross-react with chTim1- and chTim4S-transfected COS-7 cell lysates (Figure 5.17A). GG9 did not cross-react with chTim4S- and chTim4L-transfected COS-7 cell lysates, but it bound to two different bands of 50 and130 kDa in the chTim1-transfected cell lysate (Figure 5.17A), which are both bigger than the predicted size of 28 kDa.

An AEC substrate was also used in western blot analysis to determine protein products in each chTim-transfected COS-7 cell lysate, as shown in Figure 5.17B. Differently to previous observations, anti-chTim1 mAb GG9 specifically recognised only a 50 kDa band of chTim1 and a 75 kDa band for chTim4S using anti-chTim4

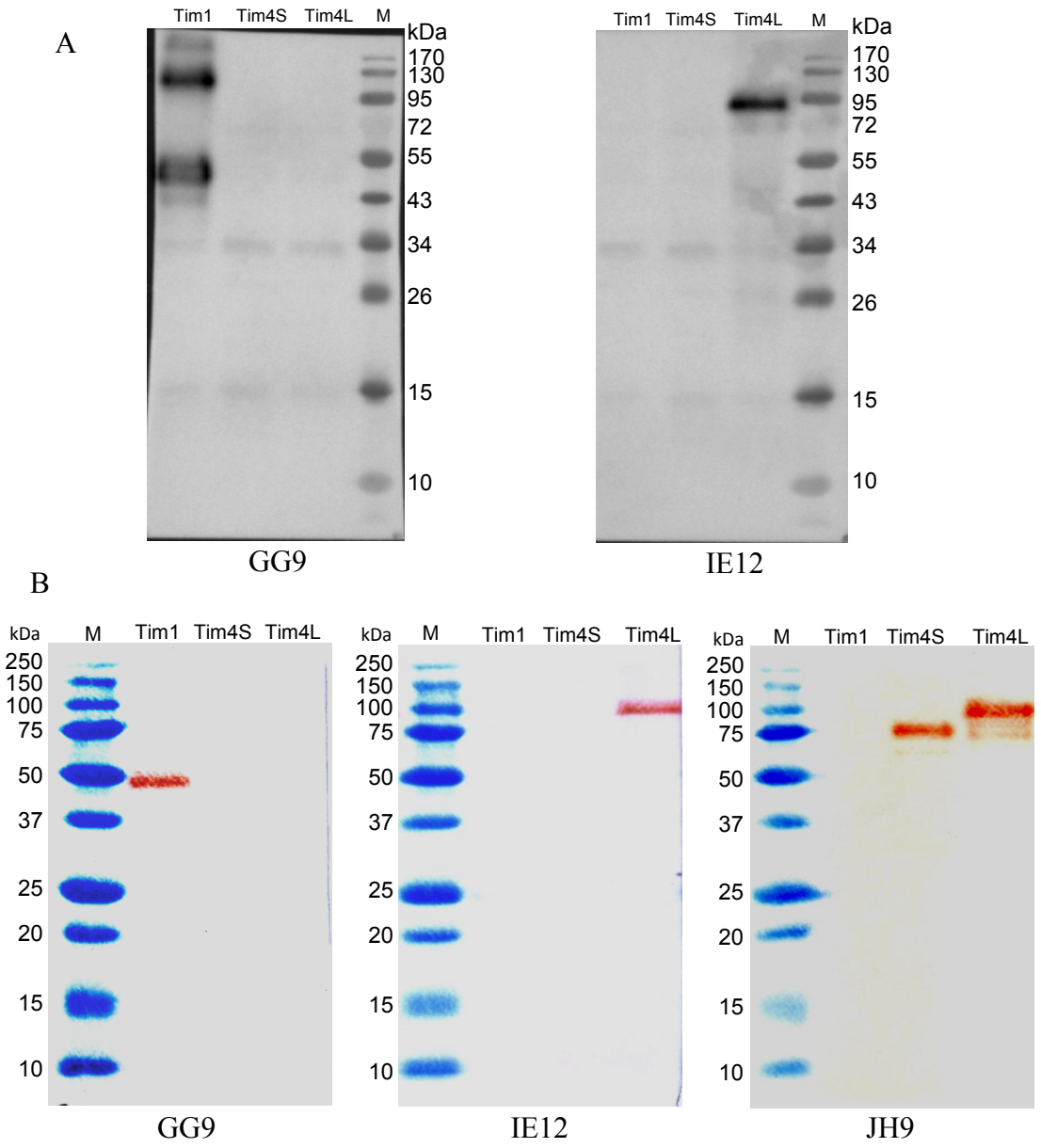


Figure 5.17. Expression of full length chTim molecules by transfected COS-7 cells, as detected by western blot. COS-7 cell lysates containing full length chTim1, chTim4S or chTim4L proteins were blotted onto nitrocellulose membranes and probed with mAbs GG9, IE12 and JH9, as labelled beneath each picture. ECL kit (A) and AEC substrate (B) were used to detect bound antibodies. Tim1 = chTim1-transfected cell lysate, Tim4S = chTim4S-transfected cell lysate, Tim4L = chTim4L-transfected cell lysate, M = Pageruler prestained protein ladder (Fermentas, A) and Precision Plus protein standard (all blue, BioRad, B).

mAb JH9. A major 95 kDa band for chTim4L protein was detected by both anti-chTim4L-hinge (IE12) and anti-chTim4S (JH9) mAbs.

5.3.7 Specificity of anti-chTim mAbs characterised with stably transfected CHO cells

To determine the ability of anti-chTim mAbs to recognise native proteins constitutively expressed on the cell surface, CHO cells were stably transfected with vectors expressing full-length chTim cDNAs, as described in section 5.2.9. Monoclonal cultures of stably transfected CHO cells were then immunostained with anti-chTim mAbs, followed by flow cytometric analysis, as shown in Figure 5.18. The anti-chTim1 mAbs, GG9, CF3 and EG7, specifically bound to chTim1-transfected CHO cells, but not to chTim4S- and chTim4L-transfected cells, compared with negative controls, which included cell controls, and isotype-matched control mAbs, ILA12 or GRi3.1. The anti-chTim4L-hinge mAbs, IE12, EA3, DD9 and AE7, specifically recognised chTim4L-transfected CHO cells, but did not cross-react with chTim4S- and chTim1-transfected cells. However, one anti-chTim4L-hinge mAb, BE6, weakly bound to both chTim4L- and chTim4S-transfected CHO cells. Anti-chTim4S mAbs recognised both chTim4S- and chTim4L-transfected CHO cells, but did not cross-react with chTim1-transfected cells. However, staining of chTim4S-transfected CHO cells with anti-chTim4S mAbs showed a broad distribution of fluorescence intensity, suggesting that the chTim4S protein is not homogeneously expressed by each cell, which may be caused by a heterogeneous cell culture. Consistent with previous results, JF1 showed different patterns in immunostaining chTim4S- and chTim4L-transfected cells, strongly to the former, but relatively less to the latter. These results showed that anti-chTim mAbs can

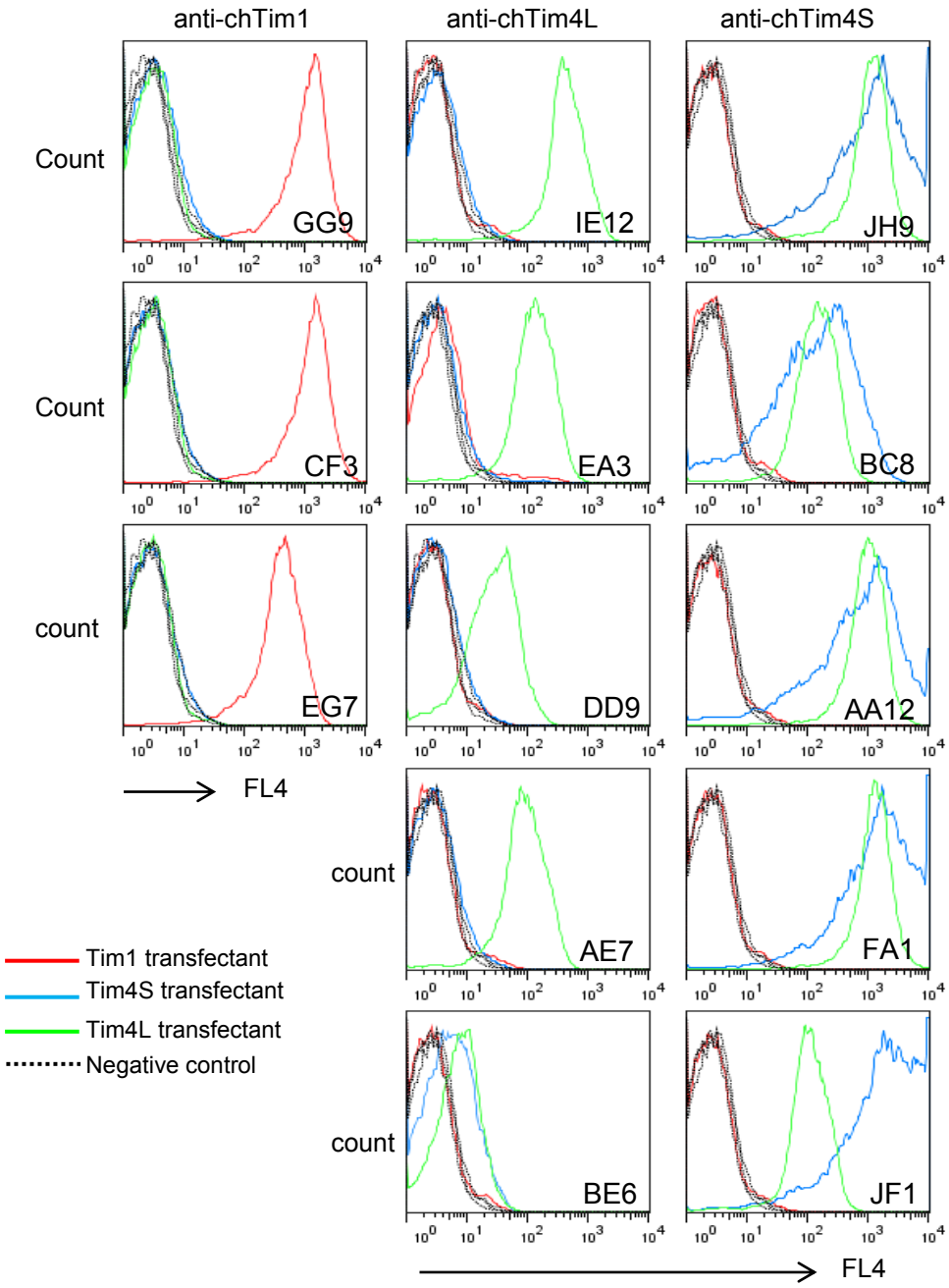


Figure 5.18. Immunostaining of stably transfected CHO cells. CHO cells were stably transfected with full-length cDNAs of chTim1 (red), chTim4S (blue) or chTim4L (green), followed by immnostaining with anti-chTim1 (GG9, CF3 and EG7), anti-chTim4L-hinge (IE12, EA3, DD9, AE7 and BE6) or anti-chTim4S (JH9, BC8, AA12, FA1 and JF1) mAbs. Dotted black lines represent negative controls, which are corresponding isotype mAbs, ILA12 (IgG2a) or GRi3.1 (IgG1). The bound primary antibody was detected by an Alexa fluor 647-labelled goat anti-mouse IgG Ab (1:1,000 dilution), shown as FL4 in x-axis. All primary mAbs were diluted at 1 µg/ml, 50 µl of them for immunestaining.

recognised their own antigens in their native form expressed on the cell surface.

To determine the strength of recognition by the anti-chTim mAbs of their own antigens, the previous immunostaining results of transfected CHO were compared, as shown in Figure 5.19. The shift in distance of the fluorescence intensity peaks illustrates the binding efficiency of the mAb. Among the anti-chTim1 mAbs, GG9 and CF3 are higher than EG7 (Figure 5.19A). For the anti-chTim4L-hinge mAbs, the descending order is IE12 > EA3 > AE7 > DD9 > BE6 (Figure 5.19B). The anti-chTim4S mAbs appear to have different binding patterns to the chTim4S and chTim4L antigens. JH9, AA12 and FA1 strongly bind to both chTim4L- and chTim4S-tranfected CHO cells (Figures 5.17C and D); but compared to them, BC8 and JF1 bind chTim4L-tranfected CHO cells more weakly. BC8 is slightly weaker than the other anti-chTim4S mAbs in immunostaing chTim4S-tranfected CHO cells.

5.4 Discussion

The immune response is more potent when the phylogenetic distance between the antigen source and the immune system involved increases. Therefore, it is an advantage to choose the mouse to generate anti-chTim antibodies, because the chicken is evolutionarily distant from the mouse and the chTim molecules also share very low homology with their murine equivalents (discussed in Chapter 3). The molecular size of the antigen also influences its immunogenicity. Therefore, a strategy of fusing a human Ig Fc tag of large molecular weight to the chTim proteins not only facilitates the purification of recombinant fusion proteins, but also increases the immunogenicity of the chTim molecule. As expected, the immunisation of mice with Ig-tagged chTim fusion proteins strongly provoked antigen-specific immune

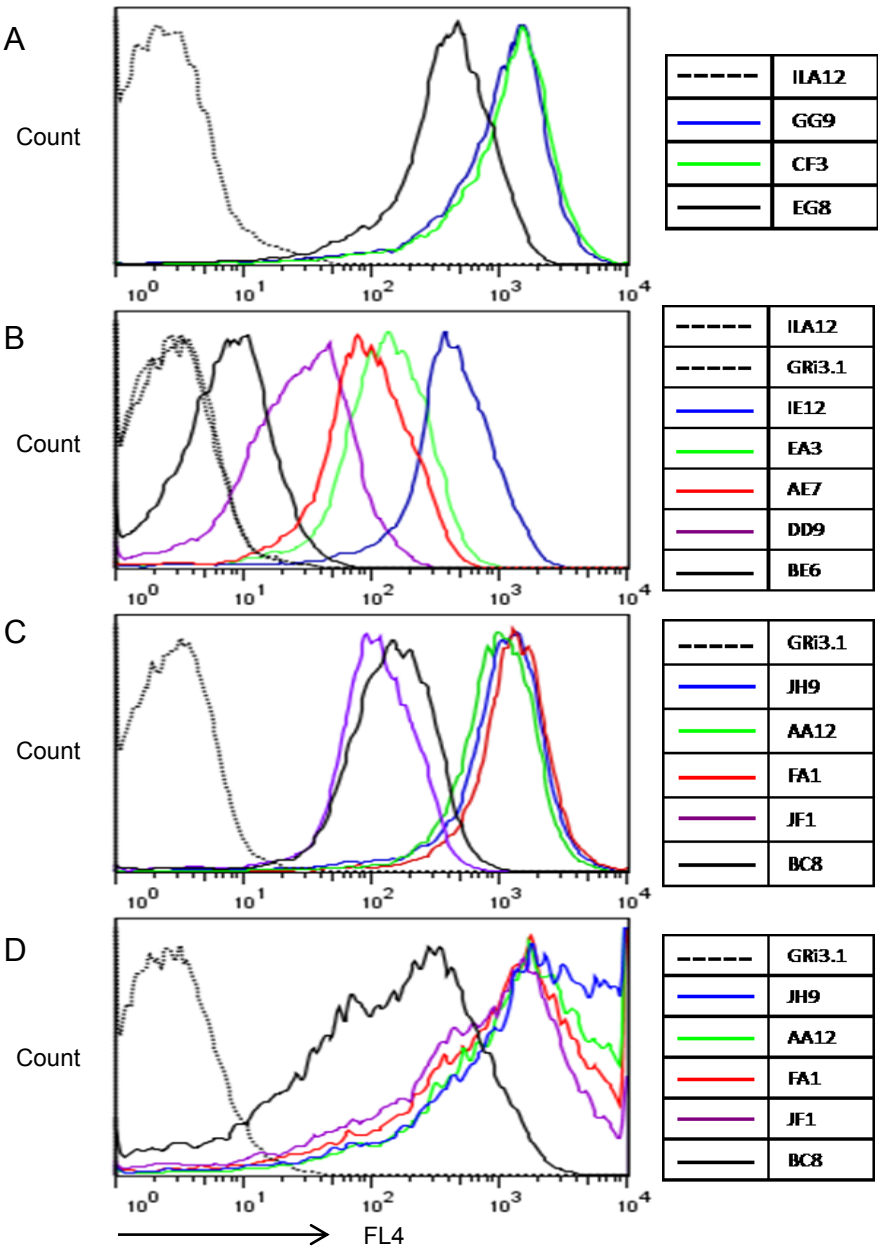


Figure 5.19. Characterisation of anti-chTim mAbs with immunostaining of stably transfected CHO cells. (A). Immunostaining of chTim1-transfected CHO cells with anti-chTim1. (B). Immunostaining of chTim4L-transfected CHO cells with anti-chTim4L-hinge. (C). Immunostaining of chTim4L-transfected CHO cells with anti-chTim4S. (D). Immunostaining of chTim4S-transfected CHO cells with anti-chTim4S. The mAbs and corresponding coloured lines are listed in the legends. Negative controls are isotype mAbs, ILA12 (IgG2a) or GRi3.1 (IgG1), as shown in dotted black lines. The bound primary Ab was detected by an Alexa fluor 647-labelled goat anti-mouse Ig Ab (1:1,000 dilution), shown as FL4 in x-axis. All primary mAbs were diluted at 1 µg/ml, 50 µl of them for immunestaining.

responses in the majority of mice. However, a big drawback of immunisation with Ig fusion protein is a concurrent anti-Ig response which produces high background effects during hybridoma screening. To diminish detection of Ig responses, modified methods were used, such as competitive ELISA or the use of His6-tagged proteins in indirect ELISA, and these efficiently improved the accuracy of the screening results.

The anti-chTim antibodies specifically recognised their own antigen in ELISA assays, but the chTim4L-extracellular-Ig fusion protein was excluded, because the soluble protein was not available (discussed in Chapter 3). To characterise the ability of anti-chTim4L-hinge mAbs to recognise full-length chTim4L proteins, COS-7 cells were transfected with vector expressing full-length chTim4L cDNA. The transfected cells were fixed and permeablised prior to staining with antibodies. The confocal results (Figure 5.12) revealed that anti-chTim4L-hinge mAbs can recognise chTim4L protein, but the staining was condensed around the nucleus. A consistent result was observed for soluble chTim4L-Ig fusion protein (Chapter 3), where the protein was trapped in the ER instead of being secreted into the supernatant.

Anti-chTim mAbs were also used to immunostain COS-7 cells transfected with vector expressing full-length chTim1, chTim4S and chTim4L cDNAs (Figures 5.13-5.15). Anti-chTim1 and -chTim4S mAbs recognised the antigens expressed by transfected COS-7 cells and the positively-stained cells, like scrambled eggs, showed the colour homogeneously spread across the entire cell. However, in the cells positively stained by the anti-chTim4L-hinge mAbs, the peripheral colour was weaker than the areas surrounding the nucleus, which are consistent with the previous confocal results, suggesting the protein is concentrated in the central region.

Murine Tim proteins are expressed in intracellular vesicles of transfected cell lines (Santiago et al., 2007a; 2007b). This intracellular expression was explained as a subsequence of the PS binding site in the Tim molecules. With mutation of this site, the protein preferentially expressed on the cell surface rather than intracellularly (Santiago et al., 2007a). PMA and ionomycin treatment can also induce protein trafficking to the cell surface; PMA and ionomycin drive an increase of intracellular calcium and subsequently facilitate the exposure of PS-bound Tim to the cell surface (Santiago et al., 2007a). As discussed in Chapter 3, chTim4L has two PS binding sites in the molecule. Therefore, they possibly cause full-length chTim4L to be expressed intracellularly instead of on the cell surface.

The molecular size of the full length chTim proteins was determined by western blot analysis of the transfected COS-7 cell lysates. In the chTim1-transfected cell lysate, two bands at 50 and 130 kDa were detected and both are bigger than the predicted chTim1 molecular weight of 28 kDa, suggesting they may be highly glycosylated chTim1 proteins. Multiple molecular sizes of mammalian Tim1 protein have been observed. Human activated T cell lysate contained three bands at 40, 70 and 110 kDa, which all are bigger than the predicted size of 36 kDa (Mesri et al., 2006). Rat postischemic kidney extract also contained three sizes of Kim1 (Tim1) protein at 40, 50 and 70-80 kDa, all bigger than the predicted size of 31.3 kDa, but two sizes of Kim1 proteins, at 40 and 63 kDa, were expressed by transfected COS cells (Ichimura et al., 1998). The multiple sizes of Tim1 protein were explained to be as a result of glycosylation, and different cell types can alter protein modification because of their different glycosylation pathways (Ichimura et al., 1998). AEC

substrate revealed a 50 kDa band to be the major product of full-length chTim1 protein expressed by transfected COS-7 cells (Figure 5.17).

In immunostaining of transfected COS-7 cells, anti-chTim4L-hinge mAbs recognised the chTim4L antigen, but not the chTim4S antigen. However, anti-chTim4S mAbs recognised both chTim4S and chTim4L antigens. Therefore, how can one distinguish chTim4S from chTim4L with anti-chTim4S mAbs? Western blot was used to analyse the presence of full-length chTim4S and chTim4L proteins in transfected COS-7 cell lysates. The anti-chTim4S mAb, JH9, recognised not only a major 75 kDa band in the chTim4S-transfected cell lysate, but also a 95 kDa band in the chTim4L-transfected cell lysate, a similar product being detected by the anti-chTim4L-hinge mAb, IE12 (Figure 5.17). This knowledge will be used in Chapter 6 to detect and distinguish chTim4S and chTim4L expression on chicken primary immune cells. However, 33 kDa bands in the chTim4S- and chTim4L-transfected cell lysates were also detected by the anti-chTim4S mAbs (Figure 5.16), suggesting the full-length chTim4S and chTim4L proteins may be in different glycosylation states.

To address if the anti-chTim mAbs specifically recognise their native form of antigens, stably transfected CHO cell lines were established. ChTim proteins, constitutively expressed on the cell surface, were analysed by flow cytometry. Consistent with ELISA and immunostaining of transfected COS-7 cells results, the anti-chTim1 and -chTim4L antibodies specifically recognised their own antigen expressed on the surface of transfected CHO cells, and anti-chTim4S antibodies not only bound to chTim4S-transfected CHO cells but also cross-reacted with chTim4L-transfected CHO cells (Figure 5.18). The antibody binding efficiency (Figure 5.19)

was also analysed and the resultant ranking was consistent with the results from ELISA and immunostaining of transfected COS-7 cells.

Based on the characterisation of the anti-chTim mAbs, including antibody specificity, binding efficiency and recognition of different forms of antigen, GG9 (anti-chTim1), JH9 (anti-chTim4S) and IE12 (anti-Tim4L-hinge) were chosen to study chTim molecule expression on chicken primary cells, as described in Chapter 6.

Chapter 6. Expression of chTim molecules on chicken primary cells.

6.1 Introduction

Tim molecules are membrane-anchored type I proteins and play immunological and physiological roles through interaction with their receptor expressed on the target cell surface. The Tim1 protein is preferentially expressed on Th2 cells to regulate the proliferation and immune function of these cells, and on tubular epithelial cells following kidney injury to clear dead cell debris, because Tim1 is also a receptor for PS. Tim1 also plays divergent roles on other immune cells. For example, Tim1 and Tim3 proteins are constitutively expressed on mast cells and mast cells activated through them enhance Th2 cytokine production, which subsequently worsens autoimmune and allergic disorders (Nakae et al., 2007). Tim1 expressed on invariant NKT cells enhances the cellular production of IL-4 and inhibits the production of IFN-γ in the presence of TCR stimulation (Kim et al., 2010). Tim1 protein is highly expressed on splenic myeloid DCs and activated DCs, through Tim1 signalling, subsequently promote proinflammatory Th17 responses and inhibit $Foxp3^+$ T_{reg} generation (Xiao et al., 2011). Increasing expression levels of Tim1 on regulatory B cells can prolong allograft survival through regulation of production of IL-4 and IL-10 by the B cells (Ding et al., 2011). Tim1-deficient mice with a Tim1-mucin domain knock-out lack IL-10 production from regulatory B cells and are less likely to develop systemic autoimmune diseases (Xiao et al., 2012). Induction of Tim-1 expression on germinal centre B cells is B cell receptor (BCR) signalling-dependent. However, Tim1-deficient mice with an IgV domain knock-out

were used to show that Tim1 is not essential for responses of the germinal centre B cells (Wong et al., 2010). Tim1 transgenic mice with Tim1 over-expressed on T and B cells have normal B cell proliferation through BCR stimulation in comparison with cells from wild-type mice, suggesting that Tim1 may not be essential for B cell responses (Wong et al., 2010). Tim1 is expressed on $CD19^+$ B cells but not on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells from lung tissue. However, Tim1-deficient mice, lacking Tim1 expression on these cells, have the same responses in antigen-induced airway hyperreactivity as those of wild-type mice, suggesting Tim1 may not be essential for experimental allergic asthma. This finding is contrary to what was proposed in a number of other studies using neutralizing and activating antibodies. Therefore the clinical relevance of Tim1 for novel allergy therapies has been questioned (Barlow et al., 2011).

As mentioned before, Tim4 is expressed primarily on APCs, including $CD11c^+$ DCs, macrophages, and marginal metallophilic macrophages, and also on peritoneal B-1 B cells. Through its interaction with PS, Tim4 plays a role in clearance of dead cells. The biological and physiological roles of the Tim4 molecule were also studied in Tim4-deficient and transgenic mice. Tim4 transgenic mice with Tim4 overexpressed on APCs had reduced numbers of antigen-specific T cells after influenza infection, resulting in greatly reduced secondary T cell responses in *ex vivo* re-stimulation (Albacker et al., 2010). The resulting hypothesis was that Tim4-expressing phagocytic cells efficiently engulf and clear PS-expressing, antigen-specific T cells, eventually preventing T cells from proceeding into the memory cell compartment (Albacker et al., 2010). However, how overexpression of Tim4 might affect Th2 cells has not been addressed in Tim4 transgenic mice. Presumably, Tim4

can stimulate Th2 cell proliferation, but activated Th2 cells massively exposing PS on their cell surface are rapidly taken up by overexpressing-Tim4 APCs, eventually inhibiting Th2 expansion. Tim4-deficient mice have an impaired phagocytic activity for peritoneal cells, hyperactive T and B cells in response to antigen stimulation, and increased production of anti-dsDNA antibodies, a sign of an autoimmune response, suggesting that Tim4 plays a critical role in clearance of dead bodies *in vivo* and that lack of Tim4 resulted in aberrant persistence of apoptotic bodies leading to dysregulated lymphocyte activation and signs of systemic autoimmunity (Rodriguez-Manzanet et al., 2010).

The mRNAs of chTims are expressed at high levels in various chicken tissues and splenic cell subsets (Chapter 4). Anti-chTim mAbs have been generated and demonstrated to have antigen-specific activities (Chapter 5). In this Chapter, the anti-chTim mAbs will be used to address if the expression patterns of chTim proteins on chicken primary cells are similar to those of their equivalents in mammals and to understand how their immunological and physiological functions, such as phagocytic and costimulatory activities as described in Chapter 4, are undertaken by chTim-expressing chicken cells.

6.2 Materials and Methods

As described in Chapter 5, based on mAb specificity, affinity, recognition of denatured or native antigens and their epitopes, mAbs against chTim were chosen to study expression of chTim proteins on chicken primary cells. MAb GG9 was used to detect chTim1 expression. MAb JH9 was chosen to detect all chTim4 isoforms molecules, thereafter named as anti-chTim4 antibody, because its epitope was

mapped to the IgV domain of chTim4. MAb IE12 was mapped to exons 3 and 4 of chTim4; therefore, it was chosen to detect the isoforms chTim4eL, chTim4L and chTim4eS and thereafter named as anti-novel chTim4 mAb.

6.3 Results

6.3.1. Expression of chTim molecules on chicken splenocytes

To determine the expression of chTim molecules on chicken primary cells, splenocytes isolated from J line birds were stained with the anti-chTim mAbs, GG9, JH9 and IE12, as shown in Figure 6.1A. Flow cytometric analysis indicated that there was no detectable positive event following staining by any of the anti-chTim mAbs when compared with staining with the isotype mAb controls, suggesting expression levels of chTim proteins are very low or undetectable on the surface of freshly-isolated chicken splenocytes.

To determine if the chTim molecules are expressed intracellularly, permeabilised chicken splenocytes were double-stained with anti-chTim and CD4 or Bu-1 mAbs and analysed by flow cytometry. $CD4^+$ and Bu-1^+ populations were gated and anti-chTim mAb double staining of these cells measured, as shown in Figure 6.1B. Surprisingly, mAb IE12 positively stained a small population of $CD4^+$ and Bu-1^+ splenocytes in comparison with isotype-matched control mAbs, but GG9 and JH9 have no detectable staining, suggesting a sub-population of $CD4^+$ T and B cells intracellularly express novel chTim4 isoforms (chTim4eL, chTim4L and/or chTim4eS).

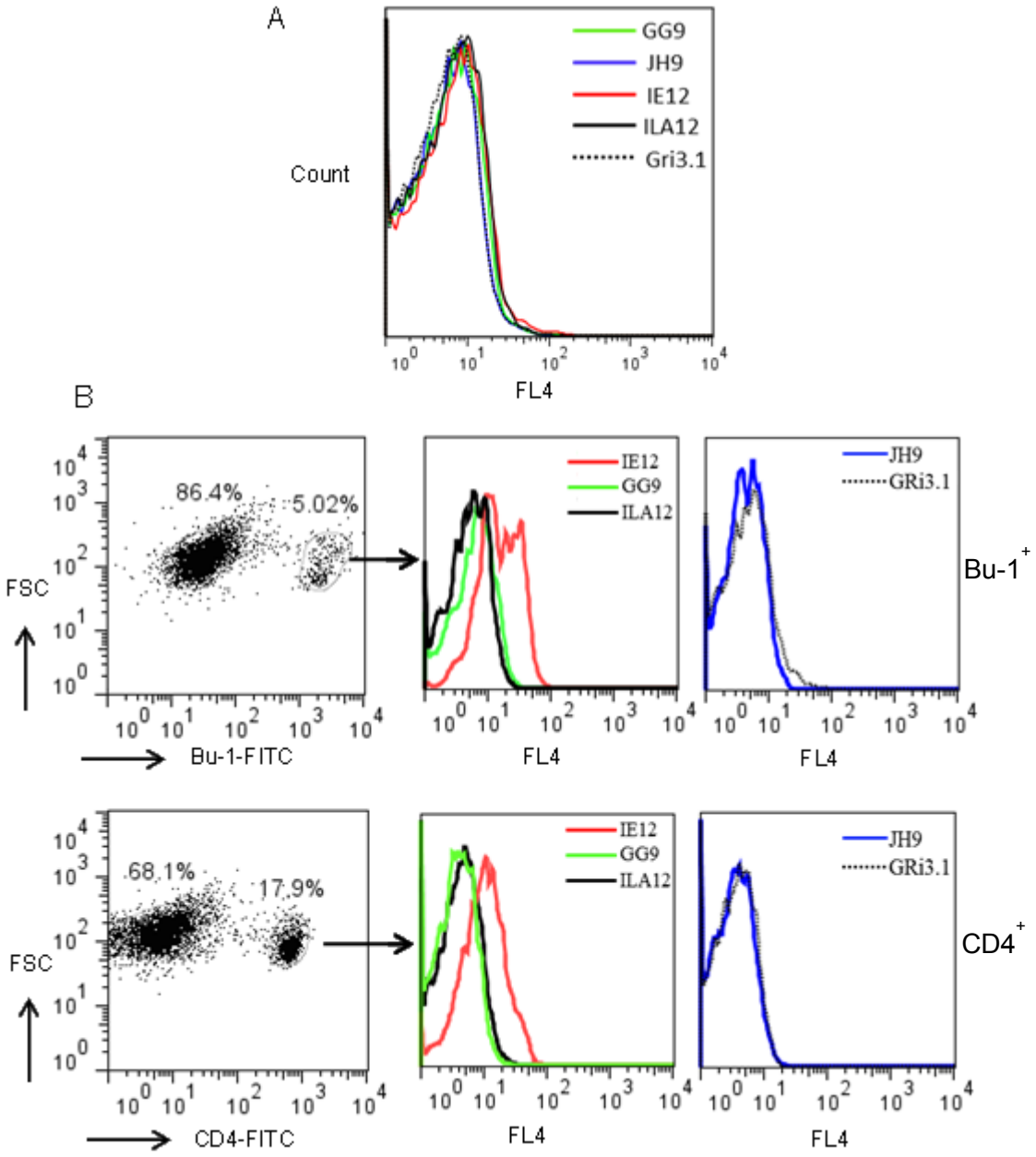


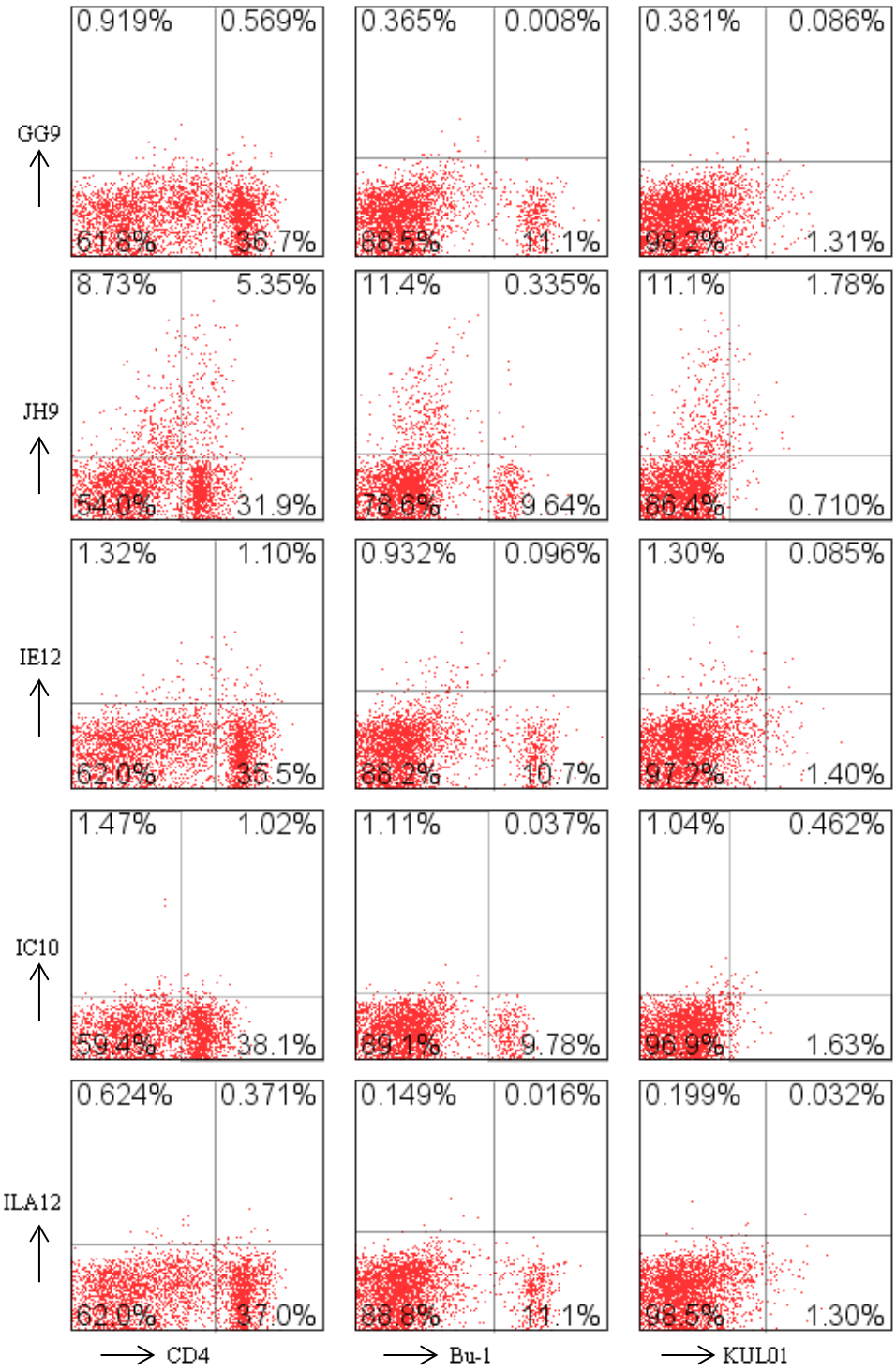
Figure 6.1. ChTim molecules expression on chicken splenocytes. (A). Surface staining of splenocytes with GG9 (anti-chTim1), JH9 (anti-chTim4), IE12 (anti-novel chTim4) and isotype controls ILA12 (IgG2a) and GRi3.1 (IgG1) mAbs. (B). Double staining of permeablised splenocytes with anti-chTim and Bu-1 (upper panel) or CD4 (bottom panel) mAbs. Bu-1$^+$ or CD4$^+$ cells are gated in dot plots (left side) and overlaid histograms analyse anti-chTim mAb double staining of the gated cells. FL4 shows the fluorescence of Alexa fluor 647. The flow cytometric results represent one experiment of three repeats.

6.3.2. Expression of chTim molecules on stimulated chicken splenocytes

To determine if activation of chicken primary cells affects expression of chTim molecules, chicken splenocytes were cultured with or without ConA, PHA and PMA for 72 h, as described in section 2.3.5.4. The cells were then double-stained with the anti-chTim mAbs, GG9 (anti-chTim1), JH9 (anti-chTim4) and IE12 (anti-novel chTim4) and antibodies to cell surface markers, including CD4 ($CD4^+$ T cells), Bu-1 (B cells) and KUL01 (macrophages). The mAbs IC10 (IgG1) and ILA12 (IgG2a) were also included as isotype controls. The resulting staining was analysed by flow cytometry, as shown in Figure 6.2.

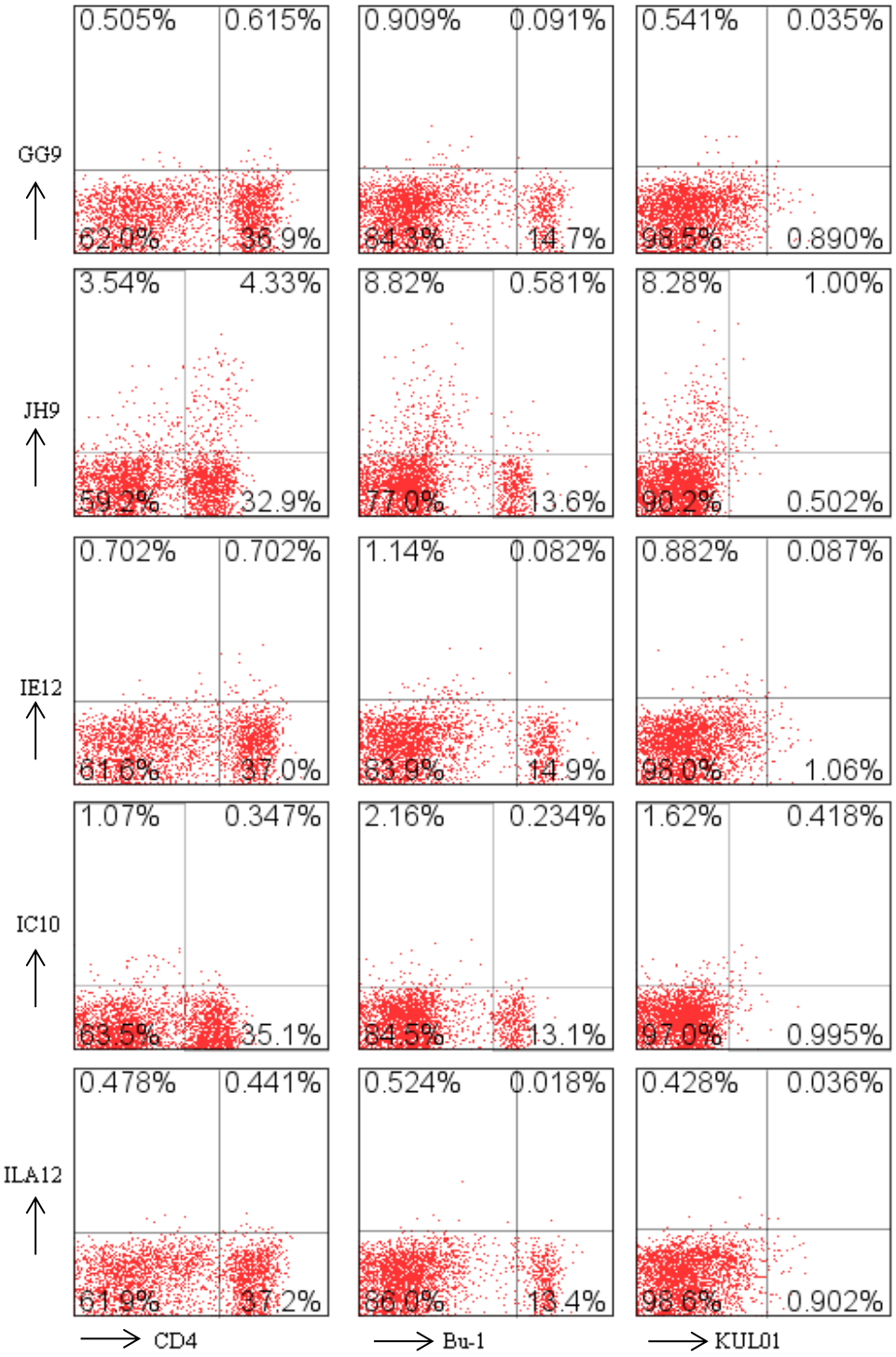
In non-stimulated splenocytes (Figure 6.2A), the mAb GG9 (anti-chTim1) did not stain splenocytes, as compared to the isotype control mAb (ILA12). However, when compared with the relevant isotype control mAb, more than 10% of the cells were stained positive (with JH9) for chTim4. One third of JH9 positive cells were $CD4^+$ cells, two thirds $CD4^-$. To see if mAb JH9 also stains B cells, these non-stimulated splenocytes were double-stained with Bu-1 and either JH9 or an isotype control mAb (IC10). Few Bu-1^+ cells were double-stained by JH9 when compared to the isotype control mAb. The mAb KUL01 was also used to double-stain these splenocytes with JH9 to address expression of chTim4 on splenic macrophages. Although the number of KUL01 positive cells was very low in non-stimulated splenocytes, the majority of the $KUL01^+$ cells were $JH9^+$ when compared to the isotype control mAb. Staining with the anti-chTim4 mAbs indicated that chTim4 proteins are expressed on the surface of cultured splenocytes, which is different to their intracellular expression on freshly-isolated chicken splenocytes

A. Non-stimulated splenocytes



B. ConA-stimulated splenocytes

C. PHA-stimulated splenocytes



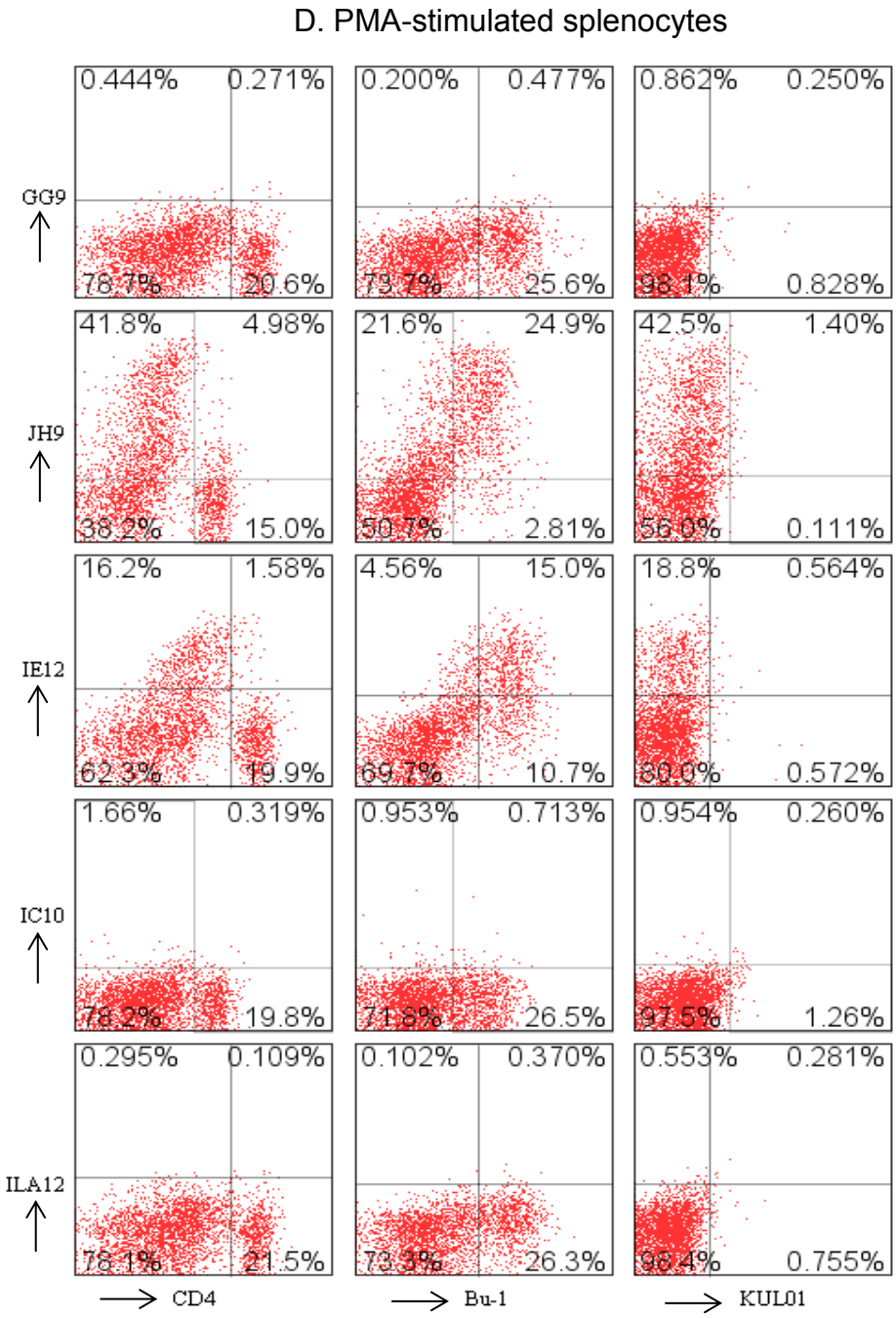


Figure 6.2. ChTim expression on conditional cultured splenocytes, stimulated with or without ConA, PHA or PMA for 72 h. The cells were then double-stained with GG9 (anti-chTim1), JH9 (anti-chTim4), IE12 (anti-novel chTim4) or isotype controls, IC10 (IgG1) and ILA12 (IgG2a), shown on the vertical axis (FL4 for Alexa fluor 647), and cell surface markers, CD4, Bu-1 or KUL01 antibody, shown on the horizontal axis (FL1 for FITC). The quadrants are drawn depending on the isotype control (horizontal quadrants) and cell surface markers (vertical quandrants). The flow cytometric results represent one experiment of three repeats.

(Figure 6.1), suggesting *in vitro* culture of splenocytes may affect expression levels of chTim4 on the cell surface. To investigate expression of novel chTim4 isoforms (chTim4eL, chTim4L and chTim4eS), the non-stimulated splenocytes were also stained with the anti-novel chTim4 mAb IE12. IE12 positively stained a higher number of cells compared to those stained with the isotype control mAb (ILA12), and, as predicted, much lower than that stained by the anti-chTim4 mAb JH9. Similarly to JH9 staining, IE12 positive cells were both $CD4^+$ and $CD4^-$, suggesting that other splenocyte subsets may also express the novel chTim4 isoforms. However, double-staining with Bu-1 or KUL01 mAb indicated little obvious IE12 positive staining when compared to the isotype control mAb (ILA12) (Figure 6.2A), suggesting low or undetectable levels of novel chTim4 isoforms are expressed on B cells and macrophages at the protein level.

In ConA- and PHA-stimulated splenocytes (Figures 6.2B and C), mAb GG9 still detected no positive cells, suggesting ConA and PHA stimulation have no obvious effects on expression of chTim1 at the protein level. Although the frequency of $JH9^+$ cells was similar to non-stimulated splenocytes, JH9 positive cells had a higher proportion of $CD4^+$ cells than non-stimulated cells, suggesting ConA- or PHA-activated $CD4^+$ splenocytes upregulated expression levels of chTim4, although both mitogens had little effect on overall expression levels of chTim4 in the total population of splenocytes. Similarly to non-stimulated cells, mAb IE12 detects few positive cells post-ConA or PHA stimulation, suggesting both mitogens had little effects on expression of the novel chTim4 isoforms.

Again, PMA-stimulated splenocytes (Figure 6.2D) were negatively stained by mAb GG9 when compared with the isotype control mAb (ILA12). However, PMA

stimulation remarkably increased the population of $JH9^+$ splenocytes in comparison with those of unstimulated and ConA- and PHA-stimulated splenocytes. Almost half of the PMA-stimulated splenocytes are $JH9^+$. To address which splenic cell subsets are the major contributors of $JH9^+$ cells, these cells were double-stained for CD4 to see if expression levels of chTim4 are upregulated on $CD4^+$ T cells. The frequency of $JH9^+CD4^+$ cells, although higher than that with the isotype control mAb (IC10), is similar to that of non-stimulated splenocytes, suggesting PMA stimulation did not dramatically affect expression levels of chTim4 protein on $CD4^+$ T cells. These cells were then double-stained with JH9 and Bu-1 mAbs to see if expression levels of chTim4 vary on B cells. Almost the entire population of $Bu\text{-}1^+$ cells was positively stained by mAb JH9 in comparison with the isotype control mAb (IC10), a remarkable increase of positive events on $Bu\text{-}1^+$ cells when compared with non-stimulated splenocytes, suggesting PMA-stimulated B cells upregulate expression levels of chTim4 protein. Double-staining with the macrophage marker, KUL01, indicated that the majority of splenic macrophages were stained by JH9, but, as expected, they were a minor contributor of $JH9^+$ cells because they are a small population in PMA-stimulated splenocytes. Notably, nearly half of $JH9^+$ cells are not B cells, staining negatively for Bu-1, suggesting other splenic cell subsets also express chTim4 protein, such as perhaps $CD8^+$ T cells.

Expression of novel chTim4 isoforms (chTim4eL, chTim4L or chTim4eS) on PMA-stimulated splenocytes was also analysed using mAb IE12. Compared with unstimulated and ConA- and PHA-stimulated splenocytes, the frequency of $IE12^+$ cells was remarkably increased, with one fifth of PMA-stimulated splenocytes staining $IE12^+$ in comparison with the isotype control mAb (ILA12). Double-staining

these cells with the B cell marker, mAb Bu-1, indicated more than half of the Bu-1$^+$ cells were also positively stained by mAb IE12, when compared with the isotype control mAb (ILA12), suggesting PMA stimulation increases expression levels of the novel chTim4 proteins on activated B cells. However, mAb IE12 stained a much lower frequency of PMA-stimulated splenocytes than JH9, consistent with the observations from non-stimulated splenocytes. Similarly to the JH9 staining, a small population of PMA-stimulated B cells did not stain with mAb IE12. To see if novel chTim4 isoforms are expressed on CD4$^+$ T cells and macrophages, these cells were double-stained with IE12 and CD4 or KUL01 mAbs. Although IE12$^+$CD4$^+$ and IE12$^+$KUL01$^+$ cells were higher in number in comparison with the isotype control mAb (ILA12), the frequencies of these cells were similar to those of unstimulated and ConA- and PHA-stimulated splenocytes, suggesting PMA stimulation has no obvious effects on expression levels of the novel chTim4 proteins (chTim4eL, chTim4L or chTim4eS) on CD4$^+$ and KUL01$^+$ splenocytes.

6.3.3. ChTim protein expression on activated chicken bursal cells

PMA stimulation increased chTim4 expression on splenic B cells. Chicken bursal cells are predominantly naïve B cells (80-90%) (Kothlow et al., 2008). To determine if activation can affect expression levels of chTim proteins on naïve B cells, bursal cells were isolated from J line birds and activated by culturing with PMA or rchCD40L for 48 h, as described in section 2.3.5.4. Expression of chTim molecules on the cell surface was detected with anti-chTim mAbs, including GG9, JH9, IE12 and isotype control mAbs ILA12 (IgG2a) and GRi3.1 (IgG1), followed by flow cytometric analysis, as shown in Figure 6.3. The Bu-1 mAb was also included, because bursal cells die very rapidly in *in vitro* culture and the Bu-1 mAb only stains

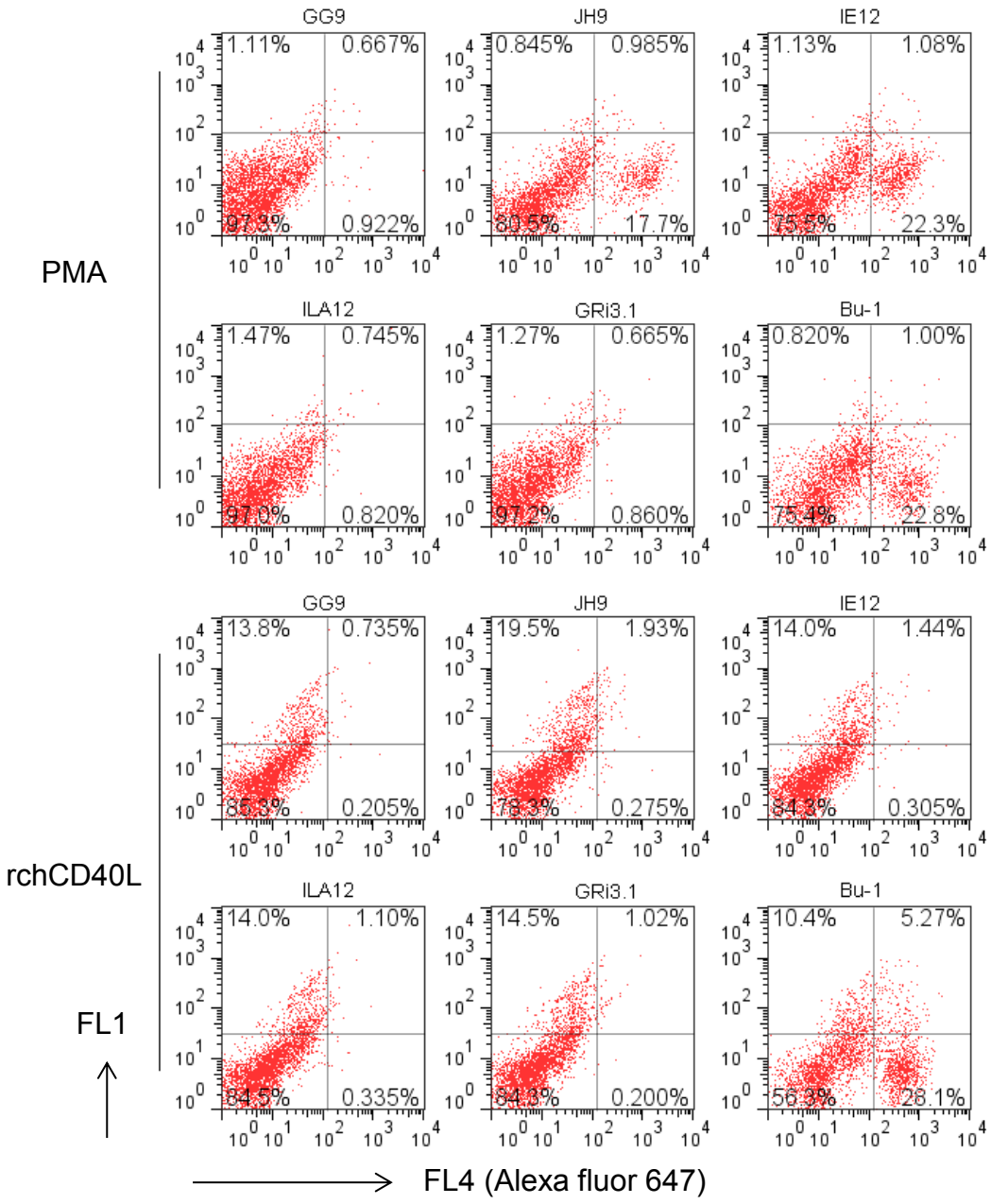


Figure 6.3. ChTim molecules expression on stimulated bursal cells. The bursal cells were stimulated with PMA or rchCD40L (labelled on left side) for 72 h. The cells were immunostained with GG9 (anti-chTim1), JH9 (anti-chTim4) and IE12 (anti-novel chTim4). Bu-1 (B cell surface marker) and isotype controls, ILA12 (IgG2a) and GRi3.1 (IgG1), as labelled above each graph, followed by Alexa fluor 647-labelled goat anti-mouse IgG antibody. Quadrants are drawn depending on isotype control mAb staining. Alexa flour 647 is on FL4 on x-axis. The y-axis shows FL1. Flow cytometric results represent one experiment of two repeats.

activated viable bursal B cells (Funk et al., 1997; Tregaskes et al., 2005; Kothlow et al., 2008). With PMA stimulation, a small population of bursal cells were Bu-1 positive, suggesting they were activated proliferating B cells. A similar population of cells were also positively stained by mAbs JH9 and IE12, suggesting chTim4 molecules are expressed on activated B cells, which is consistent with the observations from PMA-stimulated splenic B cells. However, GG9 had no detectable positive staining of PMA-stimulated bursal cells (Figure 6.3).

It was shown previously that stimulation with rchCD40L protein varies the expression pattern of chTim4 mRNA in splenocytes (Figures 3.7 and 4.3). The rchCD40L fusion protein stimulates chicken B cells to proliferate and promotes survival of activated B cells (Tregaskes et al., 2005; Kothlow et al., 2008). It was therefore used to stimulate bursal cells (predominant naïve B cells) and the expression of chTim molecules on these cells was then measured by staining with the mAbs GG9, JH9, IE12, isotype controls (ILA12 and GRi3.1) and Bu-1 (B cell marker). The resultant staining was analysed by flow cytometry. As shown in Figure 6.3, when compared with PMA stimulation, rchCD40L stimulation resulted in a higher frequency of proliferating bursal cells, which could be detected by Bu-1 mAb staining. However, rchCD40L-stimulated bursal cells were not stained by any anti-chTim mAbs (Figure 6.3). This is not consistent with the previous observation from RT-PCR analysis that rchCD40L upregulated expression levels of chTim4 mRNA (Figures 3.7 and 4.3). It is not clear if increased chTim4 mRNA expression did not translate into protein or if the chTim4 proteins were expressed intracellularly, as shown in resting splenic B cells (Figure 6.1B).

6.3.4. ChTim4 expression on bone marrow-derived DCs and macrophages

Mammalian peritoneal fluid has been used as a major source of macrophages for investigation of Tim4 expression and Tim4-dependant phagocytic activity (Miyanishi et al., 2007). Isolation of chicken macrophages from peripheral tissues (including normal peritoneal exudates) is difficult because of their low abundance. Chicken bone marrow is a source for obtaining large quantities of DCs (Wu et al., 2010b) and macrophages (Garceau et al., 2010). To determine expression of the chicken Tim4 molecules on DCs and macrophages, these cells were differentiated from bone marrow cells of J line chickens by the addition of rchIL-4 and rchGM-CSF for DCs or rchCSF-1 for macrophages, as described in sections 2.3.5.5 and 2.3.5.6. The expression profiles of chTim4 on differentiated bone marrow cells were detected by immunostaining with JH9 (anti-chTim4) and IE12 (anti-novel chTim4 isoforms) mAbs. KUL01 and isotype (ILA12 and GRi3.1) mAbs were also included as controls. The resultant staining was then analysed by flow cytometry, as shown in Figure 6.4. DCs were very weakly or negatively stained by mAbs JH9 and IE12 in comparison with isotype controls, although KUL01 positively stained the whole population of cells (Figure 6.4). By contrast, the entire population of rchCSF-1-differentiated bone marrow cells were positively stained by mAbs JH9 and IE12, and a similar population of cells were also positively stained by the mAb KUL01 (Figure 6.4). These results suggest that chTim4 molecules are expressed on immature bone marrow-derived macrophages but not on immature bone marrow-derived DCs.

To determine if activation of macrophages affects expression of chTim4, the rchCSF-1-differentiated bone marrow cells were cultured with PMA for different

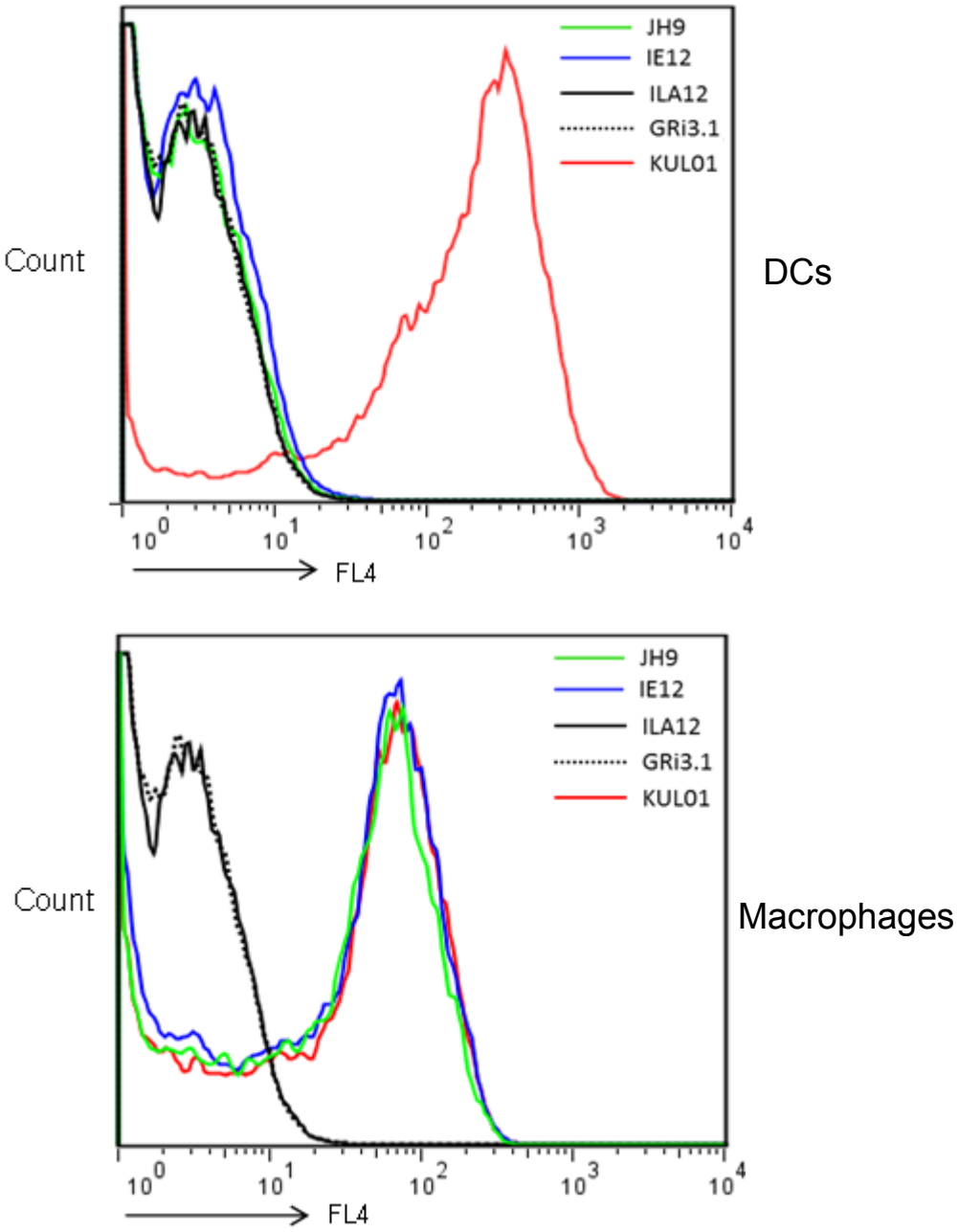


Figure 6.4. ChTim4 expression on bone marrow-derived DCs and macrophages. Bone marrow cells were cultured with rchIL4 and rchGM-CSF for DCs or rchCSF-1 for macrophages. The cells were then immunostained with JH9 (anti-chTim4), IE12 (anti-novel chTim4), KUL01 (myeloid cell surface marker) and ILA12 (IgG2a) and GRi3.1 (IgG1) as isotype controls. Secondary antibody was Alexa fluor 647-conjugated goat anti-mouse IgG, as shown in FL4 on x-axis. Y-axis indicates the positive cell count. Flow cytometric results represent one experiment of four repeats.

times. Expression of chTim4 proteins on the cell surface was detected with mAbs JH9 and IE12 and analysed by flow cytometry. KUL01 and isotype control (ILA12 and GRi3.1) mAbs were included as controls. As shown in Figure 6.5, prior to PMA stimulation (0 h), the majority of cells were positively stained by mAbs JH9, IE12 and KUL01. However, after 30 min of PMA stimulation, no positive cells were detected with mAbs JH9 and IE12, and cells remained negative until 14 h, when a very small population of cells were positive for IE12 staining. At 40 h stimulation, the number of $IE12^+$ cells seemed increased but still not clearly to be detected, and at 72 h post-PMA stimulation a clear population of $IE12^+$ cells could be detected. The effects of PMA stimulation on cell surface molecules can also be observed by detecting expression levels of KUL01 on the cell surface (Figure 6.5). Flow cytometric analysis shows that $KUL01^+$ cells appeared as a neat and sharp peak of fluorescence intensity from 0-1 h post-stimulation. At 14 h, cells stained by KUL01 had a spiky distribution, suggesting variable expression levels of KUL01 on different individual cells. At 40 h, the $KUL01^+$ cells were divided into two populations with low and intermediate expression levels of KUL01 molecule. At 72 h post-PMA stimulation, there were no cells with KUL01 expression. However, $JH9^+$ cells were not seen after 1 h post-PMA stimulation, even though the IE12 mAb positively stained a clear population of cells (Figure 6.5).

6.3.5. Western blot analysis for the presence of chTim proteins in chicken primary cell lysates using anti-chTim mAb

qRT-PCR and RT-PCR analyses showed that chTim4 mRNAs can be detected in various tissues and splenic subset cells (Figures 4.1-4.3). Flow cytometric analysis further demonstrated that the chTim4 proteins are expressed on splenocytes

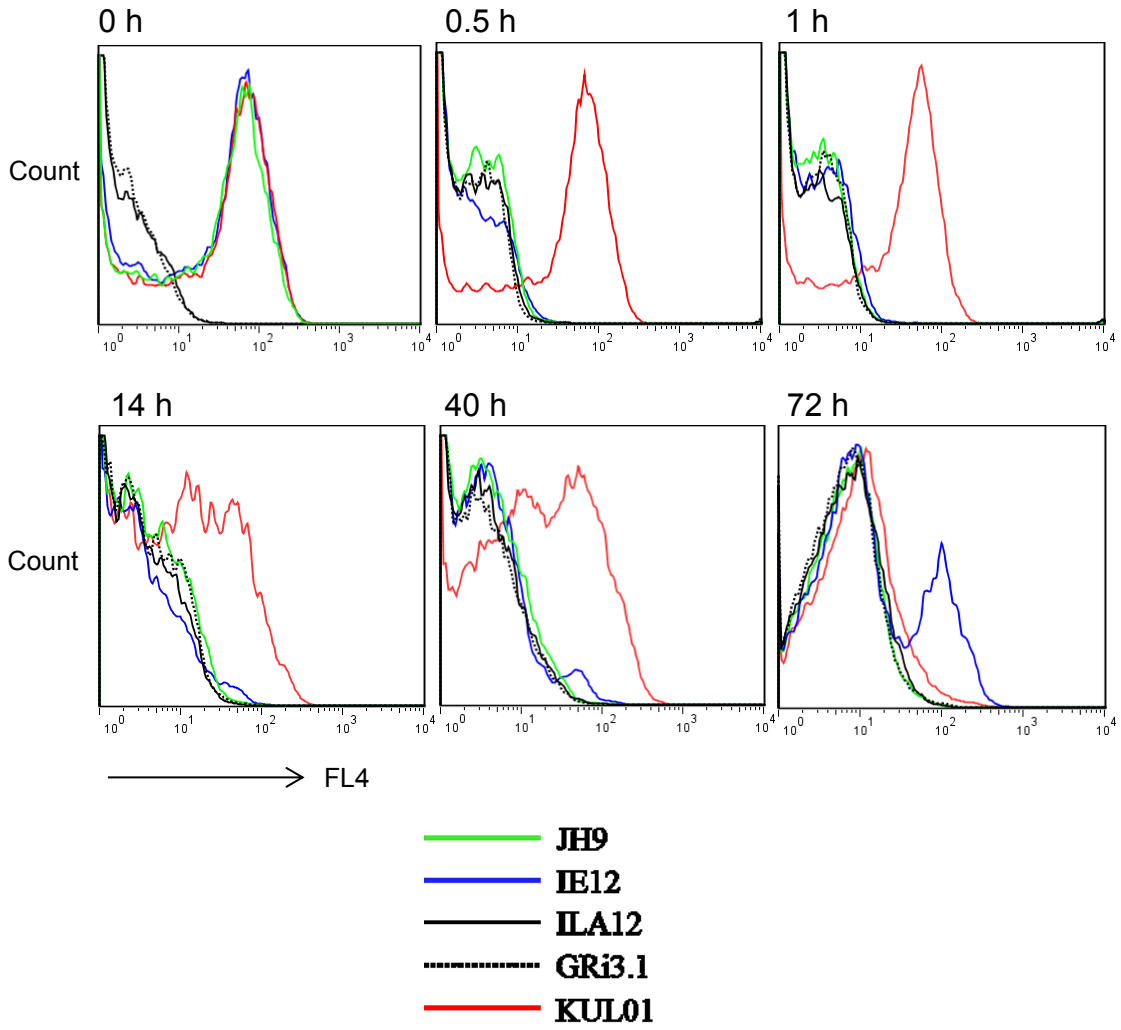


Figure 6.5. Kinetic expression of chTim4 molecules on PMA-activated macrophages. Macrophages were obtained by culturing bone marrow cells with rchCSF-1 protein for 6 days. The cells were then stimulated with PMA. The chTim4 expressed on cells were detected at different time-points by immunostaining with JH9 (anti-chTim4) and IE12 (anti-novel chTim4) mAbs. KUL01 and isotype control mAbs ILA12 (IgG2a) and GRi3.1 (IgG1) were used as control mAbs. Secondary antibody was Alexa fluor 647-conjugated goat anti-mouse IgG, as shown on FL4 on x-axis. Y-axis indicates the positive cell count. Flow cytometric results represent one experiment of three repeats.

and bursal cells (Figures 6.2 and 6.3). Western blot analysis was also used to detect the presence of chTim proteins in chicken primary cell lysates, aiming to distinguish expression of different chTim4 isoforms by their molecular weights. Chicken splenocytes, thymocytes and bursal cells were isolated from an 8-week-old J line bird (section 2.3.5) and lysed (section 5.2.8). The cell lysates were resolved on 4-12% SDS-PAGE gels (BioRad) (section 2.6.4) and transblotted to nitrocellulose membrane (section 2.6.6). ChTim proteins were probed with mAbs GG9 (anti-chTim1), JH9 (anti-chTim4) and IE12 (anti-novel chTim4). As shown in Figure 6.6, anti-chTim1 mAb, GG9 (Figures 6.6A and D) did not recognise anything. However, mAb JH9 (Figure 6.6B) recognised major bands at 45 kDa (between 37-50 kDa), which is a molecular weight band never seen in any of the chTim4S and chTim4L proteins expressed by transfected COS-7 cells (Chapter 5). MAb IE12 (Figure 6.6C), detected bands at about 100 kDa in all cell lysates, which is a similar molecular size to that of full-length chTim4L protein expressed by transfected COS-7 cells (Figure 5.15), and very faint bands at about 45 kDa (between 37-50 kDa) only in the splenocyte and thymocyte lysates. Detection with AEC substrate again identified a major product when probed by IE12 at 100 kDa in all cell lysates (Figure 6.6F), but JH9 again recognised bands at 45 kDa in all cell lysates (Figure 6.6E).

6.3.6. Western blot analysis for the presence of chTim proteins in chicken primary cell lysates using polyclonal anti-chTim serum

A major 75 kDa product of full-length chTim4S protein was detected by anti-chTim4 mAb JH9 in transfected COS-7 cell lysates (Figure 5.15), but it was not detected in the chicken primary cell lysates. The anti-chTim1 mAb, GG9, did not detect anything in primary chicken cells by either flow cytometric (Figures 6.1-6.3)

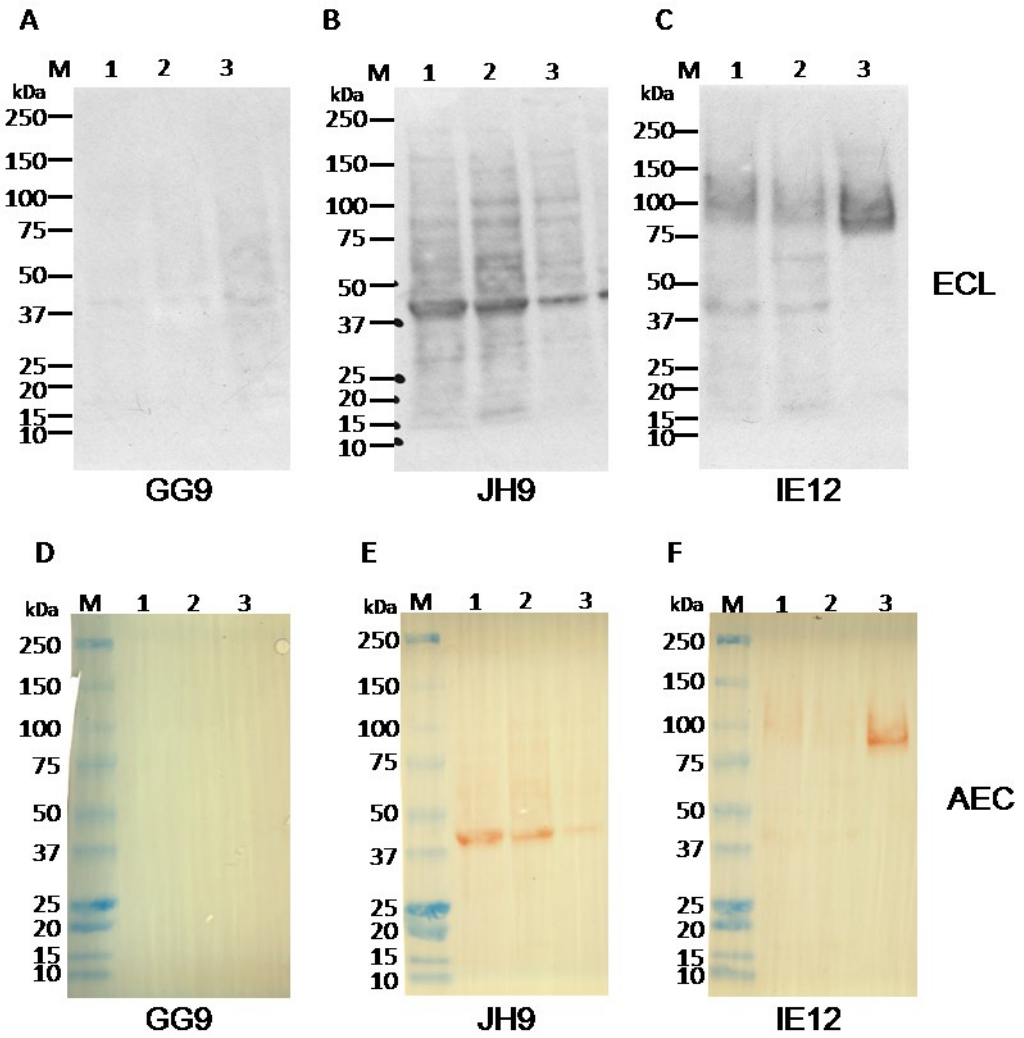


Figure 6.6. Expression of chTim proteins in chicken cells analysed by western blot. Chicken primary cell lysates were resolved on a 4-12% SDS-page gel (BioRad). The chTim proteins were probed by (A) anti-chTim1 mAb, GG9, (B) anti-chTim4 mAb, JH9 and (C) anti-novel chTim4 mAb, IE12, and followed by detection of the bound primary antibodies with a goat anti-mouse IgG-HRP antibody. HRP activity was detected by ECL reagent. The membranes were again probed with AEC substrate, as shown in (D), (E) and (F). 1 = splenocyte lysate, 2 = thymocyte lysate, 3 = bursal cell lysate, M = Precision Plus protein standard (all blue, BioRad). All cells were isolated from an 8-week-old J line chicken.

or western blot analysis (Figure 6.6). Modification of the mAb-targeted epitope may have caused the failure of these mAbs to detect chTim proteins. Therefore, to determine the expression of the chTim proteins in chicken primary cells, but avoiding any potential epitope modification problems, the chTim proteins in chicken primary cell lysates was detected by western blot using mouse anti-chTim sera obtained from the mice immunised to generate the anti-chTim mAbs in section 5.2.1. Transfected COS-7 cell lysates, containing full-length chTim4L and chTim4S proteins, were included as controls.

Anti-chTim1 serum (Figure 6.7A) identified strong bands at 72 kDa in the bursal cell and splenocyte lysates, but nothing in the thymocyte lysate. As expected, anti-chTim1 serum did not cross-react with chTim4S and chTim4L proteins in the control transfected COS-7 cell lysates. Weak 45 kDa bands were also detected by the anti-chTim1 serum in all chicken cell lysates.

Anti-chTim4S serum (Figure 6.7B) strongly bound to chTim4L and chTim4S proteins in transfected COS-7 cell lysates, with the major bands at similar molecular weights to those detected with mAbs JH9 and IE12 (Figure 5.15). Strong and clear 72 kDa bands in the bursal cell and splenocyte lysates were detected by the anti-chTim4S serum, but nothing was detected in the thymocyte lysate. As with mAb JH9 (Figures 6.6B and E), faint 45 kDa bands in all chicken cell lysates were also detected by the anti-chTim4S serum.

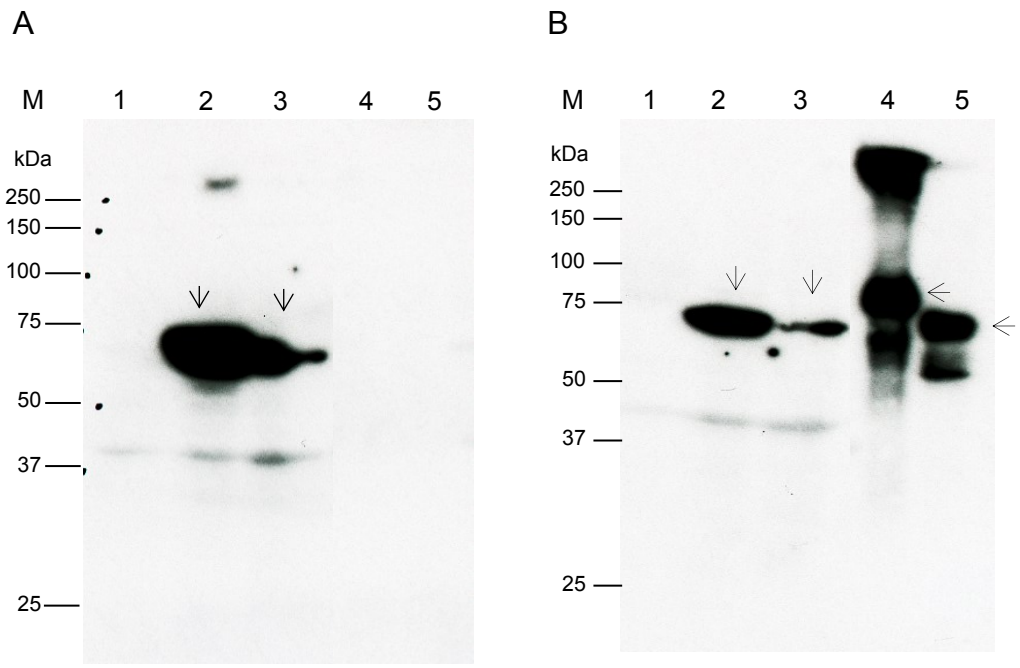


Figure 6.7. Anti-chTim sera were used to detect chTim proteins in chicken primary cells by western blot. (A) Mouse anti-chTim1 serum and (B) mouse anti-chTim4S serum were used to probe chTim proteins in cell lysates from (1) thymocytes, (2) bursal cells and (3) splenocytes, as well as transfected COS-7 cells as controls, containing (4) chTim4L or (5) chTim4S protein. M = Precision Plus protein standard (all blue, BioRad). The arrows point to the major products. All sera were diluted at 1:1000.

6.4 Discussion

The anti-chTim mAbs have been intensively characterised previously (Chapter 5) and they specifically recognise their own antigens, expressed by transfected cell lines, in native or denatured forms. Based on this knowledge, the expression of chTim molecules on chicken primary cells was examined in this Chapter.

Transcription of the chicken Tim mRNAs, as analysed by qRT-PCR, suggested they were expressed in splenic cell subsets (Figure 4.2). To determine their expression at the protein level, chicken splenocytes were stained with the anti-chTim mAbs. However, flow cytometric analysis revealed very low or undetectable expression levels of the chTim proteins on the cell surface (Figure 6.1A). To determine intracellular expression patterns of the chTim molecules in chicken primary cells, and if they were similar to those previously observed with the chTim4L fusion protein, where soluble or membrane-anchored protein was restrained inside transfected COS-7 cells (Figures 3.16, 5.12 and 5.14), splenocytes were fixed and permeabilized to detect the presence of chTim proteins with the anti-chTim mAbs. Staining with mAb IE12 for novel chTim4 proteins (chTim4eL, chTim4L or chTim4eS) (Figure 6.1B), indicated intracellular expression by a small number of $CD4^+$ and Bu-1^+ splenocytes. ChTim4 is a cell membrane-anchored protein, as described in Chapter 3; it should be expressed on the cell surface with its extracellular domain sticking out to interact with its ligands, such as PS or chTim1. Intracellular expression of chTim4L protein, as detected with flow cytometry, would limit its interaction with its ligand(s) and thereby prevent chTim4-mediated phagocytosis of dead cells or activation of T cells by interaction with Tim1. It

remains to be determined if intracellular expression of chTim4 means that chTim4-expressing cells are in a quiescent state with inactive biological roles. It is notable that the anti-chTim4 mAb JH9 recognised all isoforms of chTim4 molecules, but negatively stained $IE12^+$ splenocytes (Figure 6.1B). This issue will be discussed below.

Murine Tim4 is not expressed on T cells, but is expressed exclusively on APC (Kobayashi et al., 2007; Meyers et al., 2005a). Murine peritoneal macrophages express Tim4, but thioglycollate-elicited peritoneal macrophages lack Tim4 expression (Miyanishi et al., 2007). Murine peritoneal B-1 cells express Tim4, but conventional B cells from the peritoneum have no detectable expression of Tim4 (Rodriguez-Manzanet et al., 2010). In murine splenocytes, conventional DCs, $CD4^+$ and $CD8^+$ DCs and macrophages express Tim4. LPS and CD40L stimulation can upregulate Tim4 expression levels on splenic DCs. However, naïve and activated splenic T and B cells lack Tim4 expression (Mizui et al., 2008). In the chicken, differently to mammals, a small number of chicken $CD4^+$ and Bu-1^+ splenocytes express novel chTim4 isoforms (chTim4eL, chTim4L or chTim4eS) intracellularly. These novel chTim4-expressing $CD4^+$ splenocytes could be $CD4^+$ DCs. Bu-1^+ macrophages can be found in the chicken spleen (Houssaint et al., 1989). It remains to be determined if the novel chTim4-expressing Bu-1^+ splenocytes are B cells or macrophages, although Bu-1 is normally used as B cell marker.

To determine if cell activation affects the expression of the chTim molecules, chicken splenocytes were stimulated *in vitro* with or without ConA, PHA or PMA. ConA and PHA are lectins, which non-specifically activate T cells, but have little effect on B cells (Stobo et al., 1972; Greaves et al., 1974). PMA was also included

for stimulation of T and B cells in a relatively specific way, because it is a small organic compound which diffuses through the cell membrane into the cytoplasm, where it directly activates the protein kinase C (PKC) pathway (Mizuguchi et al., 1986; Chatila et al., 1989; Baier et al., 2009).

Without any mitogen stimulation, *in vitro* culture alone dramatically increased the frequency of $JH9^+$ splenocytes (Figure 6.2). In comparison with freshly-isolated splenocytes, chTim4 expression was upregulated on the surface of cultured splenocytes. This increased expression of chTim4 probably means that, during *in vitro* culture of the cells, chTim4-associated functions are activated. For example, phagocytes or cytotoxic lymphocytes could sense dying cells and initiate their physiological activity by increasing expression of chTim4 on the cell surface. To determine which types of cells express chTim4 protein, the cultured splenocytes were double-stained with anti-chTim4 mAbs and cell surface markers. Flow cytometry results indicated that $CD4^+$ splenocytes can be stained by the anti-chTim4 mAb, JH9 (detecting all chTim4 isoforms), but a small population of $CD4^-$ splenocytes were also stained by JH9. However, B cells did not express chTim4 on their surface because the JH9 mAb did not stain $Bu\text{-}1^+$ cells. Splenic macrophages were positively stained by JH9, but their number was much lower than that of $JH9^+CD4^-$ cells. These $JH9^+CD4^-$ splenocytes could be other splenocyte subsets, such as $CD8^+$ splenocytes. ConA or PHA stimulation did not result in massive increases in $JH9^+$ cells and had little effects on B ($Bu\text{-}1^+$) cells and macrophages ($KUL01^+$). Interestingly, stimulation caused a dramatically shift in the $JH9^+$ population to $CD4^+$ cells and resulted in fewer $JH9^+$ $CD4^-$ cells (Figure 6.2). These results may suggest inducible expression of chTim4 on activated $CD4^+$ T cells,

which is not seen in mammals, implying chTim4 may play a different role on these chicken cells to their equivalent in mammals.

Surprisingly, PMA stimulation resulted in approximately 50% of splenocytes staining positive with JH9, and also a notable increase in $IE12^{+}$ cells, suggesting expression levels of chTim4 are upregulated on these cells. Double staining of PMA-stimulated cells revealed that activated splenic B cells predominantly express chTim4, but not $CD4^{+}$ T cells. The PMA-targeted PKC kinases are a gene family of nine isotypes. According to their structure and cofactor dependency, they can be classified as conventional PKC isotypes α, β and γ; novel PKC isotypes δ, ε, η and θ; and the atypical PKC isotypes ζ and τ (Steinberg, 2008; Baier et al., 2009). Conventional PKCs require Ca^{2+} and diacylglycerol (DAG) for activation; novel PKCs are Ca^{2+}-independent; and atypical PKCs require neither Ca^{2+} nor DAG for activation (Steinberg, 2008; Baier et al., 2009). Members of the protein kinase C (PKC) family are crucial in T cell signalling pathways, particularly PKC α, PKC β, and PKC θ (Baier et al., 2009). Murine primary splenic B cells highly express the novel PKCs δ and ε but only weakly express the θ isoform in comparison to murine T cells, and these novel PKCs play a critical role in B cell activation (Krappmann et al., 2001). The specific expression pattern of PKC isoforms and their physiological roles in chicken immune cells is not known. In this study, the PKC pathway can enhance expression levels of chTim4 on activated B cells, and this activation may be in a Ca^{2+}-independent manner, because PMA can activate chicken B cells to upregulate chTim4 expression without the addition of ionomycin. Ionomycin, a Ca^{2+} ionophore, elevates intracellular Ca^{2+} concentration in treated cells and is normally used with PMA to activate the conventional PKC isoenzymes (Chatila et al., 1989,

Liu and Hermann, 1978). However, expression of chTim4 did not change on PMA-stimulated T cells, suggesting regulation of expression of chTim4 could be dependent on selective activation of PKC isoenzymes in chicken T and B cells.

Previous results indicated that stimulation with rchCD40L fusion protein altered chTim4 mRNA transcription in *in vitro* cultured splenocytes (Figures 3.7 and 4.3) with upregulation of chTim4L but downregulation of chTim4S. To determine if rchCD40L fusion protein also stimulates naïve B cells to express chTim4, bursal cells, which are predominantly naïve B cells, were stimulated with rchCD40L. PMA was also included to determine if it has a similar effect on naïve B cells as it did on splenic B cells. As with splenic B cells, the flow cytometry results (Figure 6.3) showed PMA-stimulated bursal cells express chTim4. However, rchCD40L-stimulated cells downregulated chTim4. It remains to be determined if inducible expression of chTim4 proteins on B cells is associated with the PMA stimulatory signalling pathway, but more weakly regulated by the CD40-CD40L pathway. PMA, an analog of diacylglycerol, can stimulate B lymphocytes through activation of PKC, sharing similar signalling events with BCR-dependent signalling pathways (Mizuguchi et al., 1986, Krappmann et al., 2001). Therefore PMA is normally used to mimic antigen receptor-mediated B cell stimulation. However, CD40 is a member of the tumour necrosis factor receptor (TNFR) superfamily, and with its engagement, downstream signal transduction involves association of TNF receptor-associated factors (TRAFs) and subsequent IκB kinase (IKK) activation (Hu et al., 1994; Ishida et al., 1995; Krappmann et al., 2001). The differences between B cell activation through PMA and cross-linking CD40 have been compared in the literatures, because some crucial constituents in the signal pathways are differentially regulated. For

example, the transcription factor E-twenty-six (Ets)-1, a key regulator of the Ig heavy chain gene enhancer, is inhibited by PMA stimulation, but CD40-mediated activation has no effect on it (Raghunandan et al., 2013); CD40-mediated B cell proliferation results in upregulation of Src kinase production and activity, Src (or c-Src), a tyrosine kinase, was originally identified as a chicken cellular homology of v-Src discovered in chicken Rous sarcoma virus, which controls tumour formation (Oppermann et al., 1979), but PMA-treated B cells have little change in Src kinase activity or protein levels (Neron et al., 2006). Alternatively, rchCD40L stimulation may result in an increased expression of chTim4 proteins but intracellularly, as with staining of freshly-isolated splenocytes (Figure 6.1B).

Inducible expression of mammalian Tim4 protein has been reported in mammalian APCs. Staphylococcal enterotoxin B enhances Tim4 expression in human DCs, which, in turn, drive Th2 cell polarisation (Liu et al., 2007). Murine Tim4 restrains expression of CD80, CD86 and MHCII on macrophages, which dampens the biological activities of macrophages in priming T cell responses, consequently leading to an attenuation of ConA-induced hepatitis (Xu et al., 2010). However, murine Tim4 is not expressed by immature DCs derived from bone marrow (Miyanishi et al., 2007). Chicken macrophages and DCs are present in low abundance in peripheral tissues and are difficult to isolate due to a lack of relevant reagents. *In vitro* culture methods yielding relative pure and high numbers of macrophages and DCs from chicken bone marrow cells were recently established (Wu et al., 2010b; Garceau et al., 2010). Based on these methods, macrophages were differentiated from chicken bone marrow cells by culture with rchCSF-1 and DCs by culture with rchGM-CSF and rchIL-4. Similarly to murine bone marrow-derived

DCs, immature chicken bone marrow-derived DCs lack expression of chTim4 (Figure 6.5). However, chTim4 proteins are expressed on immature bone marrow-derived macrophages (Figure 6.5). The differential expression of chTim4 on macrophages and DCs may suggest that macrophages, armed with this PS receptor on the cell surface, may be in immune surveillance mode to capture apoptotic cells and thus maintain host body homeostasis, whereas DCs, as professional antigen presenting cells, presenting exogenous antigens, may be less important in clearance of apoptotic host cells.

PMA is also a commonly-used activator of mammalian macrophages to stimulate them to differentiate and replicate (Greenberger et al., 1980; Huang et al., 2002). In differentiation of human primary monocytes or the monocyte cell line THP-1 into macrophages, PMA has even stronger effects than LPS (Kohro et al., 2004; Park et al., 2007; Daigneault et al., 2010). To determine if stimulation of bone marrow-derived macrophages affects the expression of chTim4 proteins, rchCSF-1-differentiated bone marrow cells were stimulated with PMA. The kinetics of chTim4 expression were measured by immunostaining the cells with anti-chTim4 mAbs, followed by flow cytometric analysis (Figure 6.6). After 30 min stimulation, $JH9^{+}$ and $IE12^{+}$ cells quickly disappeared, and this negative staining lasted until 14 h, when a very small population of cells were $IE12^{+}$. The number of $IE12^{+}$ cells slightly increased at 40 h, and at 72 h post-PMA stimulation a clear population of $IE12^{+}$ cells was detected. The kinetics of chTim4 expression may suggest that, at early stages of stimulation (30 min), the cells sense PMA stimulation and initiate internalisation of the receptor, leading to down-regulation of chTim4 expression on the cell surface. However, it is not clear if this internalisation of chTim4 is a specific or non-specific

response to PMA stimulation. Later, with continuous PMA stimulation, a small number of stimulated monocytes may differentiate into macrophages and express novel chTim4 isoforms (positively stained by the anti-novel chTim4 mAb IE12) on the cell surface, but it is not clear which novel chTim4 isoform was expressed latterly on PMA-stimulated macrophages. Alternatively, chickens may have two types of activated macrophages, as in mammals, which have two activated forms of macrophages, M1 and M2. The former are classically activated macrophages and immune effector cells that are aggressive against microbes and can engulf and digest them readily; the latter are more associated with a function in wound healing and tissue repair, but turn off activation of the immune system (Mosser et al., 2008). PMA stimulation could result in a different type of activated chicken macrophage with different expression levels of the novel chTim4 isoforms (chTim4eL, chTim4L or chTim4eS). PMA stimulation caused a decrease in expression levels of another cell surface protein, that recognised by the cell surface marker KUL01 (Figure 6.6), but in a much slower process when compared to disappearing of chTim4 expression after 30 min stimulation. Initially, the individual cells homogenously expressed KUL01, with a neat and sharp peak of fluorescent intensity. After 14 h of PMA stimulation, staining had a spiky distribution, indicating expression levels of KUL01 are variable on different cell surfaces. A further differentiation resulted in two clear fluorescence intensity peaks of KUL01 staining at 40 h. Eventually the KUL01 molecule disappeared from the cell surface at 72 h.

Efforts were also made to investigate differential expression of chTim proteins on different chicken primary cells with western blot analysis. The cells isolated were splenocytes, thymocytes and bursal cells from J line birds; splenocytes

are a heterogeneous population with the majority being resting T and B cells and a low number of APCs; thymocytes are predominantly naïve T cells and bursal cells are mainly naïve B cells. The presence of chTim proteins in these cell lysates were detected by anti-chTim mAbs (Figure 6.7).

IE12 mAb detected bands at approximately 100 kDa in all three cell lysates, which is a similar molecular weight as that of the full-length chTim4L protein (approximately 95 kDa) expressed by transfected COS-7 cells (Figure 5.15), suggesting the chTim4L protein may be expressed by chicken primary cells. This result is consistent with flow cytometric detection, where IE12 mAb could immunostain small numbers of splenocytes (Figure 6.1B). Differential glycosylation is thought to cause multiple molecular sizes of the full-length chTim4S and chTim4L proteins observed in transfected COS-7 cells (Figure 5.14). However, none of them match the 45 kDa bands detected by the JH9 mAb in chicken primary cell lysates (Figure 6.6); it remains to be determined if 45 kDa band is chTim4eS or other chTim4 isoforms of low molecular weight.

As mentioned previously, the epitope recognised by mAb JH9 was mapped to the chTim4 IgV domain, therefore it should recognise all chTim4 isoforms, whereas mAb IE12 was designed to recognise polypeptides encoded by exons 3 and 4 and therefore it should recognise chTim4eL, chTim4L and chTim4eS. As expected, mAb JH9 could recognise chTim4L protein in transfected COS-7 and CHO cells (Figures 5.13 and 5.16). Consistently post-PMA stimulation, a higher number of splenocytes was positively stained with mAb JH9 than IE12 (Figure 6.2). However, it is intriguing that JH9 did not stain $IE12^+$ splenocytes (Figure 6.1B), nor $IE12^+$ macrophages stimulated with PMA (Figure 6.5). This may be caused by modification

of chTim4 proteins, such as O- or N-linked glycosylation, leading to mAb JH9 binding less well to chTim4. This was further demonstrated with western blot analysis, where mAb JH9 bound less well to a high molecular weight form of chTim4, but preferentially to a small molecular weight form of chTim4 (Figure 6.6).

To reduce the possibility of the monoclonal antibodies failing to detect proteins of interest, anti-chTim sera from mice immunised for generation of anti-chTim mAbs were used to probe the lysates from thymocyte, splenocyte and bursal cells by western blot analysis (Figure 6.7). The chTim4S and chTim4L proteins in transfected COS-7 cell lysates were used as controls. Similarly to the western blot analysis using anti-chTim4S mAbs in Chapter 5, polyclonal anti-chTim4S serum could strongly detect chTim4S and chTim4L proteins in transfected COS-7 cell lysates, with major products of approximately 75 and 95 kDa respectively. Anti-chTim4S serum detected very strong bands at 72 kDa in bursal cell and splenocyte lysates, a similar molecular weight to full length chTim4S protein in control transfected COS-7 cell lysates, but also detected very weak bands at 45 kDa in all chicken cell lysates (Figure 6.7), a molecular weight consistent with detection of mAb JH9 in all chicken primary cell lysates (Figure 6.6). Anti-chTim4S serum detected strong 72 and weak 45 kDa bands, but not 100 kDa bands as detected with mAb IE12 in chicken primary cell lysates (Figure 6.6), suggesting chTim4S but not chTim4L may be predominantly expressed and chTim4eS or other chTim4 isoforms of low molecular weight expressed at low or intermediate levels.

Anti-chTim1 serum detected very strong 72 kDa bands in bursal cell and splenocyte lysates. However, these bands are different in molecule weight to those of chTim1 proteins in transfected COS-7 cell lysates detected by the anti-chTim1 mAb

(Figure 5.17), suggesting that chicken primary cells and transfected COS-7 cells probably express differentially glycosylated chTim1 proteins. This differential glycosylation of chTim1 may also cause a modification of the epitope recognised by the anti-chTim1 mAb GG9, resulting in GG9 not detecting chTim1 protein in chicken cell lysates (Figure 6.6). Although a similar pattern of molecular weight bands in bursal cell and splenocyte lysates were detected by both anti-chTim1 and anti-chTim4S sera, the former detected much stronger 72 kDa bands than the latter and the former did not cross-react with chTim4S and chTim4L proteins in control transfected COS-7 cell lysates (Figure 6.7C), suggesting 72 kDa bands may genuinely represent the chTim1 protein.

Chapter 7. General discussion.

This project aims to determine if the chicken has Tim family molecules and if their biological and immunological activities are similar to those of mammalian counterparts. MAbs against chicken Tim molecules were also generated for further investigation of their expression patterns on chicken immune cell subsets, again in comparison to those seen in mammals.

As described previously, mammalian Tim family molecules have specific expression patterns on immune cells, especially Tim1 on Th2 cells and Tim4 on APCs. Tim4 is a natural ligand of Tim1; through Tim1-Tim4 interactions, APCs drive Th2 cell proliferation. The chicken Th1/Th2 paradigm has been studied for many years, but little is known about specific Th1/Th2 cell surface molecules. Studying the chicken Tim family molecules will help to understand how the immune responses of chicken T helper subsets are regulated through their surface molecules and also provide immunological tools to further study these cells.

7.1 Chicken Th2-biased immune responses

As described in Chapter 1, the Th1/Th2 paradigm extends to the chicken to enable it to clear infections with a variety of pathogens, such as Th1 cells secrete the key Th1 cytokine, IFN-γ, to clear infections with intracellular pathogens, including bacterial (Kogut et al., 2005b; Beeckman et al., 2010) and viral (Degen et al, 2005; Eldaghayes et al, 2006) infections. Th2 cells produce the signature cytokines, IL-4, IL-13 and IL-5 (Avery et al., 2004) to clear infections with extracellular pathogens, such as parasites (Rothwell et al., 2004; Powell et al., 2009; Andersen et al., 2013).

In mammals, Th2 responses minimize the virulence of helminth infections by activating other intestinal cells, such as intestinal epithelial cells (IECs) and goblet cells, to repair damaged tissue, and reduce helminth numbers in the intestinal tract by increasing intestinal fluid volume, with the help of increased intestinal muscle contraction, to flush the worms out of the gut (Girgis et al., 2013). Mammalian Th2 responses are typified by high IgE titres. During the disease process, activated Th2 cells increase Th2 cytokine production. IL-13 and IL-4, in cooperation with the CD40-CD40L pathway, activate B cells and upregulate IgE production that can in turn activate the cells that express Fc receptors (FcRs), such as basophils, eosinophils, and mast cells, to amplify the type 2 response by producing more IL-4 (Girgis et al., 2013). However, a deleterious effect of overwhelming Th2 responses is increased allergies and asthma in mammals.

The chicken has few Th2 cytokine-targeted cells - eosinophils, mast cells and basophils - in peripheral blood. The eotaxins, critical chemokines for recruit of eosinophils in allergical responses, and their receptors are missing in the chicken (Kaiser et al., 2005). Chickens lack IgE. Th2-associated allergies are unknown for birds (Vegad and Katiyar, 1995). IL-5 is switched off during Th2 responses (Powell et al., 2009). All these facts suggest that the chicken Th2 immune responses have differentiated from those of mammals during their 300-million-year separation from a common ancestor. Interestingly, the chicken still maintains Tim1 and Tim4.

7.2 Identification and characterisation of chicken Tim cDNAs

Two clusters of chicken EST sequences homologous to mammalian Tim1 and Tim4 molecules were found by Blast searches of the chicken genome and EST

databases (Chapter 3). The chicken Tim family only has two genes, chTim1 and chTim4. There were no significant matches elsewhere in the chicken genome. The chicken Tim locus has conserved synteny with mammals, flanked by several conserved marker genes. Analysis of the Tim locus in other avian species indicated that the turkey has a similar Tim family to the chicken, with two members, Tim1 and Tim4, whereas the zebra finch has a Tim3-like gene as well as Tim1 and Tim4, suggesting domestic and wild birds may have differently evolved their Tim families.

Based on information from the EST sequences, chTim1 and chTim4 cDNAs were amplified by RT-PCR. Like mammalian Tim1, chTim1 is a type I protein with a signal peptide, IgV, mucin and transmembrane domains and a cytoplasmic tail. The IgV domain of chTim1 molecule has 6 conserved cysteines, presumably involved in forming the correct secondary structure and a PS-binding motif associated with phagocytosis of dead cells. The chTim1 mucin domain has large numbers of serine, threonine and proline residues (T/S/P-rich), which are potential O-linked glycosylation sites. The cytoplasmic tail of chTim1 has a tyrosine kinase phosphorylation motif, which suggests that chTim1 is involved in signal transduction, as are its mammalian equivalents.

Differently to mammalian Tim4, two isoforms of chTim4 - chTim4S and chTim4L - were initially cloned from RNA from a line 7_2 bird. Like chTim1, the chTim4S cDNA encodes a type I protein with a signal peptide, IgV, mucin and transmembrane domains and a cytoplasmic tail. The IgV domain also has a PS-binding motif and the mucin domain is T/S/P-rich. However, chTim4 does not have a tyrosine kinase phosphorylation motif, suggesting it does not signal. The chTim4S mRNA is encoded by 9 exons, lacking exons 3, 4 and 5 of chTim4L. The chTim4L

molecule, encoded by 12 exons, is almost identical to chTim4S in the signal peptide, mucin and transmembrane domains and the cytoplasmic tail. However, exons 2 and 5 of chTim4L encode two almost identical IgV domains, which are linked by a short hinge encoded by exons 3 and 4. Comparison of chTim4L cDNA with chicken genomic sequence by Blast searches indicated that either exons 4 or 8 of chTim4L were matched to two regions in chromosome 13 in an overlapping pattern. Further analysis indicated exons 4 and 8 are highly analogous. The intronic sequences flanked them were also analysed, including intron 3 (between exons 3 and 4), intron 4 (exons 4 and 5), intron 7 (exons 7 and 8) and intron 8 (exons 8 and 9). Interestingly, intron 3 and 7 are highly homologous at their 3' ends. Intron 4 and 8 are also highly homologous at their 5' ends. Splicing of an exon is controlled by multiple elements, including the 3' acceptor splice site at the 3' end of the upstream intron and the 5' donor splice site at the 5' end of the downstream intron, as well as elements such as exonic splice enhancers and exonic silencers (Reed et al., 1986). The similarity of exons 4 and 8 of chTim4 and the proximal intronic sequences upstream and downstream of them suggests that both exons may have similar chances to be spliced during pre-mRNA splicing, implying different chTim4 splice variants should be present. As predicted, a new isoform of chTim4 was cloned from splenic cDNA from a J line bird. This new chTim4 variant is even shorter than chTim4S, and was named chTim4-extra-short (chTimeS), encoded by 8 exons with almost identical cDNA sequences to those of chTim4L, but lacking exons 5, 6, 7 and 8 of chTim4L.

Alternatively spliced mRNA are found in multiple mammalian cell surface molecules, such as human CD80 (Inobe et al., 1996), CD86 (Jeannin et al., 2000), CD28 (Magistrelli et al., 1999a), CTLA-4 (Magistrelli et al., 1999b), CD44 (Konig et

al., 1998), CD83 (Dudziak et al., 2005), Fas (Jenkins et al., 2000) and murine Fas ligand (Ayroldi et al., 1999) and Tim3 (Geng et al., 2006). Although each of them is generated by different mechanisms, the alternative splicing results in soluble and membrane-bound forms for all of them. The soluble proteins generally antagonise the binding of the membrane-bound forms to their ligands. It remains to be determined if chTim4 also has a soluble form. If the excluded exons (5, 6, 7 and 8) of chTim4 could be spliced to exon 1 (which encodes signal peptide), a soluble form of chTim4 would be produced. This soluble form could also compete with membrane-bound chTim4 for binding to its ligand, because it contains an IgV domain encoded by exon 5 and binding sites for the chTim4 ligand(s) on the IgV domain have been shown by flow cytometric analysis (Chapter 4). However, this splice form has not yet been demonstrated.

Three chTim4 variants were identified thus far. Are there more chTim4 variants? When the original RT-PCR gel picture for chTim4S and chTim4L cloning was re-examined (Figure 3.3), there was a very faint band close to but larger than the chTim4L band, implying a larger isoform of chTim4 which was ignored previously, due to its low abundance. In mammals, alternative splice events for certain molecules can vary their biological activities, but the transcriptional abundance of certain splice variants is highly associated with the tissue or cell types which undertake these activities (Kalsotra et al., 2011). Therefore, it is a reasonable hypothesis that some types of chicken cells or stimulated cells may abundantly express this predicted chTim4 variant. An attempt was then made to amplify it by RT-PCR from different RNA samples, including spleen and rchCD40L-stimulated splenocytes from a J line chicken. A new isoform of chTim4 was eventually identified from rchCD40L-

stimulated adherent splenocytes (Chapter 3). As predicted, this isoform is even longer than chTim4L and was named chTim4-extra-long isoform (chTim4eL). It is encoded by the same 12 exons as chTim4L, but exon 3 is 198 bp longer at the 5' end than that encoding chTim4L.

Therefore, four chTim4 splice variants have been identified. How is the alternative splicing of chTim4 mRNA regulated? There are 4 basic models of alternative splicing, based on global analysis of the human and mouse genomes (Matlin et al., 2005). 1) Exon skipping, where an exon or exons may be spliced out of the primary transcript or retained. This is the most common model in mammalian pre-mRNA splicing. 2) Mutually exclusive exons, where one of two exons is retained in mRNAs after splicing, but not both. 3) Alternative donor sites, where an alternative 5' splice junction (donor site) is used, changing the 3' boundary of the upstream exon. 4) Alternative acceptor sites, where an alternative 3' splice junction (acceptor site) is used, changing the 5' boundary of the downstream exon. From these models, chTim4S may be a result of exon skipping (model 1), splicing out exons 3, 4 and 5 of chTim4 and rejoining between exons 2 and 6. ChTim4L may use an alternative 3' acceptor site (model 4) in exon 3, resulting in a shorter exon 3 to rejoin to exon 2 when compared to chTim4eL.

However, RT-PCR analysis indicated that chTim4S is a constitutive form of chTim4 because it is predominantly expressed in normal spleen tissue and variety of splenocyte subsets isolated from normal chickens (Chapter 3 and 4). Stimulation with rchCD40L downregulated expression levels of chTim4S in splenocytes, but increased expression levels of chTim4L in these cells (Chapter 3). ChTim4eL seems to be present in adherent splenocytes but may require rchCD40L induction (Chapter

3). ChTim4eS expression is even lower in normal spleen tissue (Chapter 3). Exon 3 of chTim4eL is longer than in the other chTim4 variants. Elements, such as an exonic splice enhancer (ESE), may be present in the long form of exon 3, driving splicing of the shorter version in normal circumstances; however, stimulation (such as with rchCD40L) may trigger inhibitory elements, such as an exonic splice silencer (ESS), in some cell type (such as adherent splenocytes) to prevent exon 3 being spliced in the middle, resulting in the chTim4eL transcript.

ChTim4eS mRNA is at low abundance in normal spleen tissue, suggesting it may be a rare alternative splicing event. Although chTim4eS also has a short exon 3, the exclusion of exons 5, 6, 7 and 8 means that the chTim4 splicing mechanisms are much more complicated. The homology between exons 4 and 8 and the proximal intronic sequences surrounding them could be a factor in the alternative chTim4eS splicing.

The IgV domain of chTim4 interacts with its ligands, whereas the mucin domain has no particular ligands but seems to enhance interaction between IgV and its ligands, as described in Chapter 4. Through alternative mRNA splicing, a single chTim4 gene results in at least four membrane-anchored proteins. ChTim4S is probably the basic form of chTim4, with roles similar to those of mammalian Tim4 molecules. ChTim4L and chTim4eL both have two IgV domains, and stimulation can upregulate their mRNA transcription. Does an extra IgV domain mean that chTim4L and chTim4eL have more effective biological roles than those of chTim4S and chTim4eS in emergency situations, such as increased phagocytosis of dead cells caused by microbial infection or increased induction of T cell responses? A longer exon 3 in chTim4eL results in a longer hinge between the two IgV domains than that

of chTim4L. Does the longer hinge in chTim4eL mean a more flexible IgV domain at the NH_2-terminus, resulting in a higher binding affinity for its ligand than chTim4L?

7.3 Biological roles of the chTim molecules

7.3.1 ChTims act as PS receptors to mediate phagocytosis of dead cells

The chicken Tim molecules are the first PS receptors to be identified in this species. Specific interaction between chTim and PS was demonstrated by *in vitro* protein-lipid overlay assays using chTim4S- and chTim1-Ig fusion proteins (Chapter 4). Dying chicken cells expose PS on their cell surfaces, demonstrated by stimulation of splenocytes with high doses of ConA. Flow cytometric analysis indicated that the stimulated cells were strongly stained by a PS indicator, annexin V. ChTim4S-Ig can bind to ConA-stimulated chicken splenocytes, whereas pre-blocking with PS dramatically reduced chTim4S binding, suggesting the chTim4S molecule can recognise PS exposed on apoptotic chicken cells. Physiologically, chTim molecules binding to PS enhance dead cell phagocytosis. ChTim1- and chTim4-mediated phagocytic activity was also demonstrated with transfected fibroblast cells (3T3), where overexpression of chTim proteins on the surface of transfected cells resulted in increased uptake of apoptotic cells.

Similarly to mammalian Tim4, the chTim4 molecule is expressed on the surface of chicken splenic and bone marrow-derived macrophages, suggesting a similar role for chTim4 to that of mammalian Tim4 in clearance of dead cells by macrophages through interaction with its ligand PS on dead cells. A variety of

apoptotic cell recognition receptors have been documented in mammals, falling into two categories: one involved in tethering the apoptotic cell and the other in triggering an uptake signal related to macropinocytosis. However, apoptotic cell receptors, such as CD36, $\alpha_v\beta5$ integrin, CD14 and CD68, only tether aged-erythrocytes but promote little internalisation. PS receptors (PSRs) alone induce neither tethering nor uptake of PS-exposed erythrocytes. When tethering and PS receptors work together, dead cells can be effectively ingested by phagocytes. This is the so-called "tethering and tickling" model proposed by Hoffmann et al. (2001), where the "tethering" receptors help phagocytes capture dead cells through recognition of their ligands, and ligation of PSR on phagocytes then delivers a "tickle" signal, which stimulates the internalization of apoptotic cells.

Controversially, as a PSR, the Tim4 molecule lacks a cytoplasmic motif for signal transduction. Tim4 showed a "tethering" role in phagocytosis of apoptotic cells in a murine model proposed by Toda et al. (2011), where over-expression of Tim4 alone on the surface of Ba/F3 cells, a mouse pro-B cell line which has no phagocytic activity, efficiently bound apoptotic cells in a PS-dependent manner but did not engulf them; however, co-expressing Tim4 and the integrin $\alpha_v\beta3$ in transfected Ba/F3 cells showed increased binding and engulfment of apoptotic cells in the presence of milk fat globule epidermal growth factor VIII (MFG-E8), a secreted protein that can bind PS and integrin $\alpha_v\beta3$, suggesting a "tickling" role for phagocytotic signal transduction. However, it can not be excluded that Tim4 binds an unknown molecule to form a complex for transduction of engulfment signals (Toda et al., 2011).

ChTim1 also specifically binds to PS, suggesting it is a PS receptor. Overexpression of chTim1 on the surface of 3T3 cells also resulted in an enhanced uptake of apoptotic cells. However, Tim1-mediated phagocytosis of dead cell bodies has only been described on renal proximal tubular epithelial cells, where Tim1 expression converts these epithelial cells into phagocytes for the repair process after kidney injury (Bonventre et al., 2010). Mammalian Tim1 is also widely expressed on other immune cells, such as T cells, B cells and DCs, but its role is to regulate immune responses of these cells rather than phagocytic activities. For example, Tim1 expressing on activated Th2 cells positively regulates immune responses of these cells (de Souza et al., 2005). Murine DCs express Tim1, through Tim1 signalling to enhance upregulation of costimulatory molecule expression and proinflammatory cytokine production, which in turn promote effector T cell responses, but inhibit $Foxp3^{+}$ T_{reg} responses (Xiao et al, 2011). Regulatory B cells also express Tim1; acting through Tim1, these cells can be induced to promote tolerance in mice (Ding et al., 2011). The chTim1 was also expressed by T cells, B cells and DCs by qRT-PCR analysis. Presumably, it has similar immunological roles to mammalian Tim1. However, as a PS receptor, chTim1 would inevitably bind to PS-exposed cells in the *in vivo* environment, especially when the body experiences damage by pathogens or environmental effects. As a result, chTim1 may bridge immune cells to apoptotic cells and form an inflammatory milieu, either to resolve inflammation or worsen it.

7.3.2 Costimulatory activity of chTim4S

ChTim4S-Ig fusion protein alone did not significantly stimulate chicken splenocyte proliferation. However, in the presence of anti-CD3 and -CD28 antibodies, chTim4S-Ig increased splenocyte proliferation, suggesting that chTim4 is

a T cell costimulatory molecule requiring TCR and CD28 signalling. Several lines of evidence suggest that the costimulatory activities of chTim4S could be through its interaction with chTim1, as with mammalian Tim4. ChTim1 has a conserved tyrosine phosphorylation motif in its cytoplasmic domain for signal transduction (Chapter 3); chTim1 is also expressed by splenocyte subsets by qRT-PCR analysis (Chapter 4); thirdly, chTim1 protein is present in splenocyte lysates analysed by western blot using anti-chTim1 polyclonal serum, although it remains to be determined precisely which splenocyte subsets express chTim1 protein (Chapter 6).

T cell activation requires at least two signals, TCR triggering and T cell costimulatory molecules. The latter are now categorized into three families with distinct and overlapping functions, including the CD28 family (CD28, CTLA-4, ICOS and PD-1) (Chen et al., 2004), the tumour necrosis factor family (CD40L, OX40, and 4-1BB) (Croft et al., 2003) and the signalling lymphocyte activation molecule (SLAM) family (CD2, SLAM (CD150) and 2B4 (CD244)) (Veillette et al., 2003). In mammals, Tim1 costimulation functions require signals provided by the TCR and CD28 for T cell proliferation and cytokine production. Tim3, preferentially expressed on Th1 cells, is important in the development of autoimmune disease and tolerance. Tim3 may provide a negative costimulatory signal to the T cell, as the administration of a blocking antibody to Tim3 enhances the clinical and pathological severity of experimental autoimmune encephalomyelitis and Tim3-deficient mice are resistant to induction of high antigen dose tolerance. Therefore, Tim1 and Tim3 may together constitute a unique family of costimulatory molecules (Meyers et al., 2005b).

However, as described before, the chicken only has chTim1 and chTim4; the latter stimulates splenocyte proliferation probably through chTim1. Therefore, how

does the chicken immune system balance chTim1-mediated T cell activation? An inhibitory molecule may exist to antagonise chTim1-mediated T cell responses.

7.4 ChTim expression in chicken primary cells by flow cytometric analysis

qRT-PCR analysis revealed that the chTim molecules were highly expressed at the mRNA level in a variety of chicken tissues and different splenocyte subsets (Chapter 4). To investigate expression of chTim proteins in chicken immune cells, anti-chTim mAbs were generated and characterised by ELISA, western blot, immunostaining and flow cytometry, as described in Chapter 5. The anti-chTim mAbs specifically recognised different forms of the chTim antigens, including purified chTim fusion proteins, chTim proteins overexpressed on the surface of transfected CHO cells and denatured chTim proteins on fixed COS-7 cells or in COS-7 cell lysates. MAbs were raised against the hinge part of chTim4L, encoded by exons 3 and 4, by design, which specifically recognise the chTim4L antigen but not chTim4S (Chapter 5). However, the anti-chTim4S mAbs recognised chTim4L antigens, because of the high similarities of their aa sequences across the entire molecules, as described in Chapter 3. Based on the information from *in vitro* characterisation, one anti-chTim1 mAb (GG9) was then chosen to analyse the expression of chTim1 in chicken immune cells. Two mAbs against chTim4 were also chosen, including a mAb (IE12) against the hinge part of chTim4, which would detect chTim4eL, chTim4L and chTim4eS which all contain the hinge part of chTim4, named anti-novel chTim4 antibody, and a mAb (JH9) with its epitope mapped to the IgV domain of chTim4, which would detect all chTim4 isoforms, named anti-chTim4 antibody.

Staining of unstimulated primary chicken splenocytes with these anti-chTim mAbs, followed by flow cytometric analysis, indicated that there was no obvious detection of chTim molecule expression on the cell surface. However, after permeabilisation, a small population of $CD4^+$ and $Bu\text{-}1^+$ splenocytes were stained by the anti-novel chTim4 mAb (IE12), whereas the anti-chTim4 mAb (JH9) did not detect anything. This was inconsistent with the observations in Chapter 5, where JH9 always recognised the chTim4L protein, either in its native form expressed on the surface of transfected CHO cells or in its denatured form on fixed COS-7 cells and in COS-7 cell lysates. As described in Chapter 3, the chTim4 molecule has multiple potential N- and O-linked glycosylation sites. Different modification of the chTim4 protein in chicken primary cells may result in variation in the epitope recognised by the mAb JH9, but not the epitope recognised by the anti-chTim4L mAb (IE12).

Anti-chTim mAbs were also used to investigate if stimulation affects chTim expression in chicken immune cells by flow cytometric analysis (Chaptor 6). In unstimulated splenocytes, $KUL01^+$ macrophages express chTim4 on surface, consistent with mammalian Tim4 expression on macrophages. However, $CD4^+$ splenocytes also express chTim4 on the cell surface. So far, there is no evidence of Tim4 protein expression on $CD4^+$ T cells in mammals; therefore, these chTim4-expressing $CD4^+$ splenocytes could be splenic DCs, similarly to mammalian Tim4-expressing splenic $CD4^+$ DCs. Interestingly, in freshly-isolated splenocytes, a small number of cells was stained by the anti-novel chTim4 mAb IE12 intracellularly, but not by the anti-chTim4 mAb JH9, whereas in unstimulated splenocytes, both mAbs can bind to these cells on the cell surface. It could be a result that expression of the chTim4 protein on the cell surface facilitates access of mAb JH9 to its epitope.

Why is the chTim4 molecule expressed intracellularly in freshly-isolated splenocytes, but on the cell surface in cultured splenocytes? It could be a result of chTim4 proteins trafficking to the cell surface during *in vitro* culture, as described in a Tim molecule trafficking model proposed by Santiango et al. (2007a). In this model, after intracellular translation, Tim proteins are transported to the cell membrane. However, they are not directly expressed onto the surface, but bind to PS on the inner layer of the cell membrane. PS exposure to the outer layer of the membrane, due to alteration of cell membrane lipid asymmetry, results in co-transportation of Tim proteins onto the cell surface. The alteration of membrane lipid asymmetry in this model is initiated by activation of lipid scramblase, which requires influx of calcium into the cells. However, in this study, there had no any supplements, such as extra calcium iron, ionomycin etc., in growth medium to influence intracellular calcium concentration, suggesting cell membrane asymmetry may affect chTim4 expression on the cell surface, but less importantly.

ConA and PHA had no significant effects on chTim expression on splenocytes (Chapter 6). However, PMA stimulation dramatically upregulated chTim4 expression levels on splenic Bu-1^{+} B cells (Chapter 6). PMA specifically targets the PKC signalling pathway for cell activation. PMA can block anti-IgM antibodies in the activation of normal and malignant B cells, suggesting that the PKC signal pathway is shared by the BCR (Mizuguchi et al., 1986). Therefore, PMA is normally used as a substitute for anti-IgM in B cell activation. The upregulation of chTim4 on PMA-stimulated B cells suggests that the IgM-associated BCR signalling pathway may also affect chTim4 expression on activated B cells. PMA-stimulated chicken naïve B cells also upregulated chTim4 expression. Chicken bursal cells,

predominantly naïve B cells, were stimulated with PMA and rchCD40L. Flow cytometric analysis indicated that PMA-stimulated bursal cells expressed high levels of chTim4, whereas rchCD40L-stimulated bursal cells had no detectable expression of chTim4, suggesting chTim4 expression may be pathway-dependent.

$KUL01^+$ macrophages from splenocytes also expressed chTim4, as analysed by flow cytometry (Chapter 6), but with cell numbers being low it was difficult to clearly show chTim4 expression on these cells. Bone marrow cells were then cultured with rchCSF-1 to derive macrophages, or with rchGM-CSF and rchIL-4 for DCs, followed by staining with the anti-chTim mAbs. Consistently, bone marrow-derived macrophages highly expressed chTim4 on their cell surface. However, bone marrow-derived DCs had no obvious chTim4 expression. This may reflect divergent roles of chicken macrophages and DCs: perhaps chTim4 expression on macrophages enhances phagocytosis of dead cells to maintain immune tolerance; however, the lack of chTim4 expression on DCs helps them avoid uptake of dead cells, resulting in a lower risk of DCs presenting self antigen. Stimulation of bone marrow-derived macrophages with PMA resulted in rapid downregulation of chTim4 levels on the surface of these cells, suggesting immature macrophages may be more important in the phagocytosis of self/dead cells than activated macrophages (Chapter 6).

7.5 ChTim expression in chicken primary cells with western blot analysis

A competitive ELISA revealed that the three anti-chTim1 mAbs recognised overlapping or closely related epitopes (Chapter 5). The anti-chTim1 mAb GG9 did not detect chTim1 expression on chicken primary cells, either permeablised

splenocytes or ConA-, PHA- or PMA-stimulated splenocytes (Chapter 6). Western blot analysis was also attempted to detect the presence of the chTim1 protein on thymocyte, splenocyte and bursal cell lysates; again, nothing was detected by mAb GG9 (Chapter 6). These results are inconsistent with observations that GG9 strongly reacted with chTim1 protein expressed by transfected COS-7 or CHO cells either in denatured or native form (Chapter 5). It could be that the protein structure of chTim1 expressed by chicken primary cells differs from that of chTim1 expressed by cell lines (COS-7 and CHO), resulting in variation in the epitope recognised by GG9. Because the chTim1 molecule has high levels of potential N- and O -linked glycosylation sites in the protein (Chapter 3), differentially glycosylated chTim1 protein in different cell types may be due to variable levels of the enzymes which control protein modification. This phenomenon has been observed with the murine Tim1 protein, in that different cell types expressed different glycosylated forms of murine Tim1 (Ichimura et al., 1998). Therefore, a polyclonal anti-Tim1 serum was used to detect the chTim1 protein in chicken primary cell lysates with western blot analysis (Chapter 6). Differently to mAb GG9 (anti-chTim1), the polyclonal anti-chTim1 serum detected strong and clear bands at 72 kDa in both bursal cell and splenocyte lysates. By contrast, the anti-chTim1 serum did not cross-react with chTim4S or chTim4L protein in control transfected COS-7 cell lysates. The presence of chTim1 in the bursal cell lysate is consistent with the observation of its mRNA expression in bursal tissue (Chapter 4), suggesting that naïve B cells express chTim1. ChTim1 protein in splenocyte lysate could also be a consequence of chTim1 expression in different splenocyte subsets, as was measured by qRT-PCR.

To determine which chTim4 molecules are expressed in chicken primary cells, the anti-chTim4 (JH9) and anti-novel chTim4 (IE12) mAbs were used to detect the presence of chTim4 in chicken cell lysates with western blot analysis (Chapter 6). IE12 detected bands of approximately 100 kDa in thymocyte, bursal cell and splenocyte lysates, which is a molecular weight very close to that of full-length chTim4L (95 kDa) expressed by transfected COS-7 cells. However, mAb JH9 only detected 45 kDa bands in thymocyte, bursal cell and splenocyte lysates, which is a molecular weight much smaller than those of chTim4S (75 kDa) and chTim4L (95 kDa) expressed by COS-7 cells. One explanation is that chicken primary cells express other molecular weight chTim4 proteins which may not be detected by the mAbs. A polyclonal anti-chTim4S serum was then used to analyse chTim4 protein expression (Chapter 6). As expected, the anti-chTim4S serum strongly bound to chTim4S and chTim4L proteins in control transfected COS-7 cell lysates, resulting in major bands with similar molecular weights as those detected by the anti-chTim4 mAbs (JH9) (Chapter 5). Differently to JH9, besides recognition of 45 kDa bands in all chicken cell lysates, the anti-chTim4S serum also detected strong and clear bands at 72 kDa in bursal cell and splenocyte lysates, suggesting that chTim4S protein may be abundantly expressed by splenocytes and bursal cells, whereas the 45 kDa proteins could be chTim4eS or other chTim4 isoforms of low molecular weight.

Close examination of western blot detection with the anti-chTim1 or anti-chTim4S serum indicated that both of them recognised similar molecular weights and patterns of bands, especially in bursal cell and splenocyte lysates (Chapter 6). However, the 72 kDa bands detected by the anti-chTim1 serum in both cell lysates are much stronger when compared to those detected by the anti-chTim4S serum, and

the anti-chTim1 serum did not cross-react with chTim4 in control transfected COS-7 cell lysates, suggesting a 72 kDa chTim1 protein could be genuinely expressed by bursal cells (predominantly naïve B cells) and splenocytes.

7.6 Future plans

Differently to what has been reported in mammals, the chicken has several chTim4 splice variants (Chapter 3). However, RT-PCR analysis (Chapter 3) indicated that chTim4S and chTim4L are the predominant forms expressed by chicken immune cells; chTim4S was the major form expressed in a variety of splenocyte subsets from an SPF line 7_2 chicken, whereas chTim4L expression was only upregulated in rchCD40L-stimulated splenocytes. Flow cytometric analysis indicated that freshly isolated splenocytes expressed chTim4L protein intracellularly rather than on the cell surface (Chapter 6), but, after stimulation, chTim4 proteins were expressed on the surface of splenocytes (Chapter 6). Therefore, how is the chTim4 molecule regulated in chicken immune cells? One hypothesis is that chTim4S is predominantly expressed in chicken immune cells when the chicken is less immunised and lives in a hygienic environment, whereas immunisation and environmental stimulation upregulates chTim4L expression. ChTim4L protein is therefore maintained inside of the cell when the chicken's immune system is not threatened. ChTim4L can be rapidly expressed on the surface of chicken immune cells when they sense stimulation. However, this remains to be demonstrated in a disease model.

ChTim1 mRNA is expressed in chicken immune organs and cell subsets, as detected by qRT-PCR (Chapter 4). ChTim1 protein can also be detected by

polyclonal anti-chTim1 serum in bursal cell and splenocyte lysates with western blot analysis (Chapter 6). However, the anti-chTim1 mAb generated in this study did not detect chTim1 protein in primary cells, although it strongly recognised the chTim1 antigen expressed by transfected cells. It would worth generating further mAbs against chTim1, because Tim1 is so important for Th2 cell immune responses in mammals, and most of the biological roles of Tim1 in mammals were demonstrated by anti-Tim1 mAbs.

7.7 Conclusions

The chicken has fewer Tim family members than mammals, but alternative splicing of chTim4 can generate multiple transcripts which encode proteins with potentially subtle or opposing functional differences which could result in profound biological consequences, as has been demonstrated for other multiple splicing genes in mammals (Graveley et al., 2001). For example, CD45 is a receptor-like protein tyrosine phosphatase and serves as an obligate positive regulator of antigen receptor signalling to regulate T and B cell lineage development. It is encoded by 34 exons and alternative splicing leads to inclusion or exclusion of exons 4, 5, and 6 in its NH_2-terminus, resulting in eight different isoforms. Five of these have been detected at the protein level in human, and different isoforms were differentially required at different stages of T and B cell development (Hermiston et al., 2003). A cognate pair, Fas and Fas ligand (FasL), are associated with induction of cell apoptosis and both have alternative splice forms, resulting in membrane-bound and soluble proteins. The soluble forms of Fas/FasL serve as inhibitors to disrupt membrane-bound Fas signalling-induced apoptosis (Jenkins et al., 2000; Ayroldi et al., 1999). Reagents

and methods established in this project provide useful tools for further study of the biological roles of the individual chTim4 variants in healthy and infected birds.

The chicken lacks many important components of mammalian Th2-associated responses and Th2-associated allergy is rarely seen in chicken. However, Tim1 and Tim4, which both are involved in Th2 immune responses in mammals, are present in the chicken. The studies of the chTim molecules presented in this thesis will be very helpful for further dissection of the chicken's Th2 responses.

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Appendix 1 Buffers and Solutions

QIAprep®spin miniprep kit (Qiagen)

P1	50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNAse A
P2	200 mM NaOH, 1% SDS
N3	3 M KAc (pH 5.5)
PB	Aqueous mix of isopropanol and guanidine hypochlorite
PE	Aqueous mix of Tris-HCl, hydroxymethyl and amino methane
EB	10 mM Tris-HCl (pH 8.5)

EndoFree plasmid Maxi kit (Qiagen)

P1 and P2 as above

P3	3 M KAc (pH5.5)
QBT	750 mM NaCl, 50 mM MOP (pH 7.0), 15% isopropanol (v/v) and 0.15% Triton X-100 (v/v)
ER	Details not provided by manufacturer
QC	1 M NaCl, 50 mM MOPS (pH 7.0) and 15% isopropanol (v/v)
QF	1.25 M NaCl, 50 mM Tris-HCl (pH 8.5) and 15% isopropanol (v/v)
QN	1.6 M NaCl, 50 mM MOPS (pH 7.0) and 15% isopropanol (v/v)
TE	10 Mm Tris-HCl (pH 8.0) and 1 mM EDTA

QIAquick® gel extraction kit (Qiagen)

PE and EB as above

QG	Aqueous mix containing guanidine hypochlorite

RNease® mini kit (Qiagen)

RLT (RT)	Aqueous mix containing guanidine thiocyanate
RW1 (RT)	Aqueous mix containing guanidine thiocyanate
RPE (RT)	Details not provided by manufacturer
Sequencing (Buckman Coulter)	
DTCS quick start master mix	dATP, dCTP, dGTP, dTTP, ddATP, ddCTP, ddGTP, ddTTP, Thermo sequenase DNA polymerase, pyrophosphatase
CEQ sequencing reaction buffer	Tris-HCl, $MgCl_2$, pH 8.9
Stop solution	2 volumes 100 mM EDTA, 2 volumes 3 M NaOAc, 1 volume glycogen
Sample loading solution	Formamide
Miscellaneous solutions	
LB broth	10 g bacto tryptone, 5 g bacto yeast extract, 10 g NaCl, adjust to pH 7.0 with 5 M NaOH. Made up to 1 liter with Milli-Q water.
LB agar	As above with 1.5% bacto-agar
PBS	NaCl (10 g), KCl (0.25 g), Na_2HPO_4 (1.437 g) and KH_2PO_4 (0.25 g) were dissolved in 900 ml Milli-Q water, adjusted pH to 7.0 and made up to 1 litre volume with Milli-Q water.
PBST	PBS with 0.05% Tween-20
SOC medium	Dissolve 20 g bacto tryptone, 5 g bacto yeast extract, 0.5 g NaCl in 950 ml Milli-Q water, add 10 ml 250 mM KCl, 20 ml of sterile glucose and adjust pH to 7.0 with NaOH. Make final volume to 1 litre.
10× PCR buffer (Invitrogen)	200 mM Tris-HCl (pH 8.4), 500 mM KCl.
10× TBE buffer	890 mM Tris, 890 mM Boric acid, 20 mM EDTA, pH 8.0
50× HAT supplement (Life technologies)	

	A liquid mixture of sodium hypoxanthine (5 mM), aminopterin (20 μM) and thymidine (0.8 mM)
50× HT supplement (Sigma-Aldrich)	
	A liquid mixture of 100 μM hypoxanthine, 16 μM thymidine

AEC substrate kit (BD Pharmingen)

AEC buffer	Aqueous mix of sodium acetate, dimethylulfoxide, methanol, and urea peroxide
AEC chromogen	3-Amino-9-Ethylcarbazole and N, N, dimethylformamide

Mayer's hemotoxlin solution (Sigma-Aldrich)

	Haematoxylin (1.0 g/l), sodium iodate (0.2 g/l), aluminium ammonium sulfate·12 H_2O (50 g/l), chloral hydrate (50 g/l) and citric acid (1 g/l).

SDS-PAGE buffer

4× resolving buffer	1.5 M Tris/HCl, pH 8.8
4× stacking buffer	0.5 M Tris/HCl, pH 6.8

Appendix 2 Screening hybridomas producing anti-chTim1 mAbs

A total of 768 polyclonal hybridomas (eight 96-well plates) were screened by indirect ELISA.

T1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.069	0.062	0.061	0.061	0.061	0.071	0.089	**0.168**	0.101	0.096	0.078	0.062
B	0.058	0.097	0.079	**0.153**	0.07	0.091	0.118	0.076	0.069	**0.219**	0.1	0.059
C	0.06	0.066	0.092	0.074	0.068	0.082	0.072	0.068	0.066	0.065	0.068	0.058
D	0.063	0.074	0.068	0.071	0.083	0.114	**0.235**	**0.127**	0.08	0.075	0.069	0.062
E	0.059	0.063	0.088	0.082	0.084	0.089	0.082	0.086	0.105	0.087	0.079	0.062
F	0.06	0.071	0.107	**0.195**	0.078	0.103	0.094	0.091	0.098	**0.146**	0.077	0.056
G	0.064	0.065	0.089	0.082	0.089	0.087	0.082	0.093	**0.241**	0.101	0.074	0.068
H	0.105	0.064	**0.207**	**0.255**	0.114	0.114	0.079	0.1	0.081	0.074	0.082	0.062

T2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.069	0.082	0.079	**0.203**	0.1	0.097	0.086	0.093	0.087	0.079	0.072	0.07
B	0.094	0.097	0.094	0.085	0.099	0.092	0.098	0.079	0.092	0.085	0.084	0.075
C	0.075	0.084	0.109	0.107	0.094	0.1	**0.178**	0.086	0.095	0.106	0.073	0.076
D	0.081	0.11	0.1	0.1	0.109	0.095	0.091	0.115	0.084	0.076	0.075	0.068
E	0.077	**0.125**	**0.12**	0.108	0.107	0.09	0.092	0.097	0.084	0.074	0.07	0.069
F	**0.12**	0.1	0.102	0.098	0.106	0.087	0.113	0.089	0.094	0.088	**0.133**	0.07
G	0.073	0.115	0.099	0.11	0.104	0.089	0.09	0.083	0.09	0.114	0.073	0.07
H	0.069	0.077	0.115	0.074	**0.184**	0.086	0.092	0.085	0.075	0.098	0.07	0.089

T3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.11	0.096	0.105	0.116	**0.141**	**0.14**	**0.205**	**0.208**	**0.125**	0.107	0.082	0.071
B	0.076	0.107	**0.12**	0.09	0.094	0.084	0.086	0.079	0.083	0.101	0.087	0.076
C	0.073	0.075	**0.232**	**0.218**	**0.247**	**0.186**	**0.219**	**0.198**	**0.141**	0.09	0.084	0.089
D	0.077	0.1	0.08	0.093	0.106	0.095	0.106	0.117	0.101	0.094	0.086	0.075
E	0.072	0.086	0.093	0.107	0.086	0.118	0.105	0.109	0.101	0.091	0.086	0.07
F	0.108	0.091	**0.353**	0.118	**0.144**	**0.13**	**0.126**	**0.122**	**0.179**	**0.124**	0.096	**0.13**
G	0.104	0.085	0.108	**0.184**	**0.269**	0.088	0.109	**0.151**	0.105	0.1	0.082	0.072
H	0.071	0.077	0.076	0.093	**0.121**	0.105	0.114	0.102	0.087	**0.135**	**0.12**	0.071

T4	1	2	3	4	5	6	7	8	9	10	11	12
A	0.07	0.073	0.079	0.067	0.069	0.077	0.072	0.08	0.073	0.072	0.096	0.064
B	0.073	0.111	0.1	0.107	0.095	0.092	0.089	0.091	0.099	0.077	0.066	0.069
C	0.072	0.089	0.107	**0.124**	**0.184**	0.097	0.11	0.08	0.08	0.08	0.085	0.066
D	0.075	**0.125**	**0.13**	**0.12**	**0.187**	0.091	0.09	0.097	0.096	0.073	0.067	0.063
E	0.09	**0.155**	0.095	**0.13**	0.11	0.108	0.113	0.112	0.096	0.093	0.074	0.065
F	0.074	**0.152**	**0.137**	**0.349**	0.087	**0.132**	0.094	0.084	**0.124**	0.072	0.091	0.064
G	0.068	0.082	0.098	0.109	0.098	0.103	**0.16**	0.081	0.079	0.087	0.089	0.08
H	0.068	0.09	0.098	0.089	0.09	0.079	0.078	0.108	0.083	0.097	0.073	0.069

T5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.073	0.097	0.103	0.085	0.093	0.089	**0.121**	**0.14**	0.089	0.078	0.073	0.073
B	0.106	0.072	0.075	0.137	0.088	**0.136**	0.095	**0.124**	0.078	0.081	0.073	0.077
C	0.071	0.082	0.077	0.141	0.106	0.097	0.085	0.102	0.084	0.082	0.134	0.071
D	**0.12**	0.078	0.094	**0.158**	0.116	**0.374**	0.11	**0.14**	0.078	0.077	0.093	**0.131**
E	0.078	0.078	0.088	0.097	0.086	0.117	**0.127**	**0.147**	0.104	0.085	0.086	0.087
F	0.082	0.078	0.096	0.1	0.086	**0.165**	0.103	0.092	0.091	**0.25**	0.093	0.071
G	0.075	0.075	0.094	0.092	0.093	0.089	**0.426**	0.098	0.1	0.116	0.113	0.072
H	0.072	0.07	0.084	0.114	0.108	**0.133**	0.089	**0.186**	0.097	0.115	0.088	0.094

T6	1	2	3	4	5	6	7	8	9	10	11	12
A	0.118	0.07	0.075	0.076	0.088	**0.151**	0.1	0.095	0.087	0.073	0.071	0.067
B	0.075	0.08	0.088	0.099	0.084	**0.143**	0.093	0.087	0.114	0.088	0.075	0.067
C	0.072	0.076	0.097	0.097	**0.122**	0.098	0.107	**0.12**	0.088	0.078	0.095	0.069
D	0.08	0.108	0.11	0.114	0.103	0.089	0.108	0.096	**0.148**	0.081	0.096	0.068
E	0.071	0.09	0.096	**0.196**	0.095	0.088	**0.143**	**0.122**	0.105	**0.122**	0.091	0.09
F	0.072	0.108	0.093	0.088	0.088	0.094	0.097	0.117	0.109	0.115	0.075	0.073
G	0.075	0.087	0.093	0.117	0.084	0.086	0.102	0.078	0.109	0.096	0.075	0.082
H	0.088	0.074	0.083	**0.121**	0.08	**0.149**	0.083	0.086	0.085	0.112	0.069	0.068

T7	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.147**	0.067	0.068	0.08	0.076	0.074	0.099	0.073	0.166	0.088	0.099	0.072
B	0.066	0.111	0.085	**0.127**	0.097	0.084	0.078	0.117	0.086	0.074	0.071	0.085
C	0.078	0.096	0.084	0.089	0.096	**0.133**	0.086	0.082	0.087	0.101	0.077	0.07
D	**0.125**	0.086	0.082	0.102	**0.136**	0.097	0.094	0.085	0.088	0.109	0.082	0.071
E	0.071	0.081	0.078	0.098	0.112	0.095	0.09	0.113	0.082	**0.175**	0.073	0.07
F	0.07	0.083	0.091	0.09	0.123	0.119	**0.216**	0.094	0.112	0.088	0.078	0.075
G	0.074	0.078	0.08	0.111	0.094	0.082	0.094	0.092	**0.318**	0.085	0.087	0.07
H	0.071	0.077	0.086	**0.132**	0.1	0.082	0.084	0.081	0.089	0.11	0.089	0.076

T8	1	2	3	4	5	6	7	8	9	10	11	12
A	0.08	0.079	0.078	0.079	0.083	0.122	0.11	0.111	0.081	0.1	0.114	0.085
B	0.071	0.082	0.082	0.082	0.086	0.092	0.113	0.107	0.106	0.09	0.101	0.073
C	0.093	0.09	**0.154**	**0.151**	0.089	0.088	0.086	0.119	0.115	0.104	0.084	0.09
D	0.107	0.087	0.088	0.08	0.077	0.09	0.085	0.093	**0.123**	0.085	**0.127**	0.079
E	0.07	0.082	0.081	0.084	0.088	**0.145**	0.1	0.082	0.082	0.081	**0.171**	0.076
F	0.088	0.078	0.085	0.091	**0.141**	0.088	0.08	0.091	0.089	0.085	0.083	0.076
G	0.076	**0.144**	**0.142**	**0.125**	0.087	0.101	0.089	0.101	0.092	0.081	0.081	0.076
H	0.077	0.078	0.09	0.079	0.092	0.077	0.095	0.079	0.091	**0.139**	**0.127**	0.069

Note: T means test plate. The fusion was carried out with the spleen from mouse 1. ELISA was performed as described in section 5.2.4. 102 positive hybridomas, as shown in bold, were then grown up in 24-well plates.

Appendix 3 Screening hybridomas producing anti-chTim4L-hinge mAbs

A total of 768 polyclonal hybridomas (eight 96-well plates) were screened by indirect ELISA.

T1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.123	0.08	0.064	0.105	0.086	0.071	0.149	0.095	0.16	0.087	0.092	0.065
B	0.072	0.186	0.081	0.157	0.113	0.069	0.143	0.159	0.162	**0.365**	0.08	0.084
C	**0.484**	0.133	**0.449**	0.173	0.104	0.121	0.156	0.141	0.178	0.103	0.075	0.08
D	0.064	0.077	0.12	0.153	0.096	0.113	0.087	0.13	0.144	0.088	0.073	**0.382**
E	0.075	0.11	0.175	**0.212**	0.181	0.183	0.125	0.124	0.129	0.147	0.083	0.062
F	0.078	0.17	0.171	0.179	**0.256**	0.192	0.109	0.078	0.099	0.073	0.082	0.064
G	0.065	0.095	**0.192**	0.143	**0.297**	**0.219**	0.104	0.101	0.075	0.14	0.075	0.084
H	0.067	0.18	0.109	0.41	0.144	0.182	0.117	0.551	0.096	0.081	0.402	0.087

C1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.079	0.109	0.145	0.247	0.101	0.103	0.196	0.236	0.234	0.276	0.191	0.137
B	0.121	0.429	0.212	0.267	0.275	0.146	0.207	0.234	0.201	**0.599**	0.288	0.189
C	**0.591**	0.159	**0.617**	0.236	0.327	0.257	0.189	0.231	0.238	0.272	0.208	0.345
D	0.111	0.195	0.178	0.13	0.116	0.17	0.12	0.366	0.237	0.265	0.266	**0.153**
E	0.103	0.096	0.173	**0.102**	0.232	0.183	0.336	0.242	0.238	0.45	0.188	0.081
F	0.089	0.085	0.129	0.123	**0.122**	0.296	0.263	0.377	0.28	0.253	0.21	0.194
G	0.08	0.088	**0.14**	0.091	**0.407**	**0.252**	0.251	0.212	0.263	0.447	0.332	0.305
H	0.078	0.374	0.114	0.411	0.125	0.105	0.135	0.592	0.307	0.289	0.286	0.219

T2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.093	0.104	0.105	0.15	0.101	**0.235**	0.117	0.135	0.115	0.15	0.148	0.146
B	0.084	0.171	0.183	0.176	**0.566**	**0.254**	0.189	0.167	**0.239**	0.154	0.155	0.107
C	0.088	**0.288**	0.194	0.196	**0.22**	**0.413**	0.115	0.121	0.132	0.172	0.122	0.108
D	0.125	0.152	**0.194**	0.152	0.121	0.103	0.131	0.202	0.149	0.188	0.162	0.095
E	0.09	0.197	0.123	0.11	0.118	0.167	0.186	0.201	0.205	0.187	**0.536**	0.115
F	0.107	0.105	**0.617**	0.127	**0.279**	0.194	**0.214**	**0.585**	0.16	0.118	0.148	0.109
G	**0.242**	0.09	0.134	0.11	0.182	0.204	0.174	**0.204**	**0.223**	0.133	0.146	0.143
H	0.081	0.096	0.098	0.119	0.117	**0.263**	**0.237**	0.18	0.177	0.146	0.139	0.119

C2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.085	0.202	0.098	0.131	0.107	**0.332**	0.109	0.096	0.109	0.169	0.185	0.098
B	0.118	0.1	0.19	0.138	**0.53**	**0.101**	0.116	0.139	**0.304**	0.117	0.165	0.137
C	0.128	**0.497**	0.11	0.168	**0.172**	**0.574**	0.149	0.102	0.122	0.112	0.084	0.093
D	0.189	0.208	0.086	0.105	0.238	0.125	0.102	0.391	0.275	0.094	0.063	0.09
E	0.124	0.132	0.169	0.188	0.123	0.119	0.143	0.1	0.097	0.08	**0.594**	0.086
F	0.297	0.18	0.157	0.169	**0.135**	0.1	**0.092**	**0.595**	0.102	0.082	0.078	0.076
G	**0.456**	0.189	0.208	0.127	0.121	0.241	0.142	**0.092**	**0.078**	0.089	0.071	0.076
H	0.085	0.129	0.227	0.197	0.113	**0.352**	**0.279**	0.119	0.091	0.088	0.098	0.09

T3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.07	0.136	0.068	0.071	0.074	0.071	0.079	0.076	0.089	0.081	0.075	0.072
B	0.063	0.065	0.079	**0.145**	0.088	0.068	0.068	0.066	**0.256**	0.081	0.067	0.064
C	0.066	0.085	0.074	0.073	0.073	0.076	**0.396**	0.073	0.072	0.068	0.072	0.076
D	0.065	0.064	0.087	0.076	0.07	0.07	0.075	0.076	0.084	0.079	0.072	0.068
E	0.071	0.076	0.068	0.104	0.096	0.071	0.087	**0.639**	0.138	0.093	0.088	0.091
F	0.065	0.064	0.105	**0.537**	**0.155**	0.074	0.073	0.102	0.099	0.098	0.08	0.067
G	0.065	0.067	0.086	0.08	0.083	0.098	0.087	0.067	0.077	0.076	0.538	0.081
H	**0.141**	0.069	0.066	0.063	0.123	0.069	0.072	0.079	**0.176**	0.066	0.094	0.063

C3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.067	0.245	0.067	0.066	0.069	0.092	0.149	0.067	0.081	0.08	0.082	0.068
B	0.075	0.075	0.122	0.069	0.072	0.07	0.068	0.069	**0.067**	0.067	0.075	0.068
C	0.088	0.12	0.073	0.073	0.076	0.07	**0.086**	0.067	0.063	0.063	0.064	0.066
D	0.061	0.063	0.066	0.069	0.133	0.099	0.068	0.087	0.064	0.08	0.095	0.066
E	0.063	0.061	0.063	0.15	0.071	0.067	0.065	**0.108**	0.064	0.063	0.071	0.078
F	0.061	0.063	0.067	**0.068**	0.068	0.072	0.067	0.11	0.07	0.111	0.061	0.066
G	0.06	0.061	0.062	0.069	0.072	0.131	0.063	0.063	0.078	0.064	0.103	0.063
H	**0.068**	0.064	0.101	0.065	0.067	0.062	0.065	0.097	0.088	0.065	0.066	0.064

T4	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.141**	0.078	0.106	0.065	0.141	0.068	0.068	0.08	0.073	**0.429**	**0.122**	0.097
B	0.07	0.079	0.061	**0.281**	0.068	0.067	0.065	**0.213**	0.075	0.126	**0.417**	0.072
C	**0.192**	0.076	0.086	0.069	0.064	0.103	0.061	0.133	0.093	0.065	0.093	0.067
D	0.069	0.065	0.077	0.064	0.066	0.074	0.061	0.121	**0.142**	0.066	0.099	0.085
E	0.068	0.079	0.069	0.074	0.067	0.351	0.069	**0.213**	0.073	0.065	0.069	0.06
F	0.057	0.088	**0.189**	0.175	0.075	0.077	0.083	0.427	**0.314**	0.069	0.069	0.068
G	0.07	0.072	0.072	0.07	0.103	0.112	0.063	0.066	0.091	0.073	0.098	0.1
H	**0.302**	**0.125**	**0.315**	0.098	0.095	0.073	0.091	**0.327**	**0.532**	0.086	0.077	0.085

C4	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.075**	0.069	0.128	0.123	0.165	0.058	0.088	0.081	0.056	**0.072**	**0.057**	0.057
B	0.054	0.056	0.059	**0.229**	0.073	0.125	0.069	**0.39**	0.062	0.068	**0.109**	0.056
C	**0.061**	0.056	0.073	0.079	0.096	0.305	0.089	0.23	0.078	0.056	0.057	0.054
D	0.056	0.106	0.094	0.088	0.116	0.112	0.077	0.13	**0.083**	0.055	0.054	0.062
E	0.064	0.074	**0.062**	0.091	0.115	0.373	0.107	**0.116**	0.096	0.063	0.058	0.059
F	0.053	0.109	0.169	0.234	0.118	0.091	0.158	0.52	**0.346**	0.057	0.06	0.069
G	0.052	0.059	0.07	0.097	0.154	0.114	0.105	0.12	0.077	0.053	0.075	0.06
H	**0.132**	**0.057**	**0.055**	0.074	0.058	0.106	0.06	**0.323**	**0.126**	0.097	0.076	0.065

T5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.069	0.072	0.07	0.072	0.118	0.071	0.063	**0.201**	0.089	0.115	0.305	0.107
B	0.062	0.063	0.077	0.073	**0.488**	**0.515**	0.116	0.087	0.068	**0.52**	0.088	0.067
C	0.074	0.083	0.074	**0.268**	0.068	0.065	0.079	0.07	**0.42**	0.113	0.08	0.066
D	0.073	0.063	0.071	**0.193**	**0.225**	0.064	0.079	0.101	0.079	**0.415**	**0.356**	0.092
E	**0.521**	**0.245**	0.198	0.139	0.113	0.106	0.086	0.08	0.082	0.068	**0.337**	0.081
F	0.062	0.059	0.078	0.071	0.064	**0.433**	0.153	0.065	0.073	0.084	0.064	0.06
G	0.065	**0.408**	**0.522**	0.121	0.083	**0.197**	0.075	0.07	0.124	0.098	0.071	0.062
H	0.085	**0.241**	**0.18**	0.078	**0.195**	0.07	0.065	0.143	**0.305**	0.102	0.069	0.072

C5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.067	0.077	0.059	0.114	0.062	0.064	0.06	**0.126**	0.058	0.099	0.084	0.056
B	0.053	0.057	0.063	0.121	**0.109**	**0.364**	0.082	0.061	0.055	**0.064**	0.134	0.057
C	0.053	0.054	0.076	**0.068**	0.054	0.059	0.058	0.067	**0.065**	0.066	**0.054**	0.053
D	0.056	0.068	0.108	**0.104**	**0.143**	0.057	0.066	0.071	0.055	**0.441**	**0.206**	0.062
E	**0.056**	**0.084**	0.08	0.06	0.055	0.056	0.062	0.058	0.055	0.052	0.11	0.051
F	0.055	0.058	0.058	0.057	0.088	**0.492**	0.13	0.103	0.059	0.066	0.076	0.055
G	0.055	**0.419**	**0.241**	0.07	0.104	**0.131**	0.089	0.104	0.131	0.064	0.054	0.053
H	0.051	**0.076**	**0.077**	0.052	**0.131**	0.062	0.054	0.108	**0.084**	0.081	0.068	0.058

T6	1	2	3	4	5	6	7	8	9	10	11	12
A	0.071	0.092	0.125	**0.413**	0.154	0.109	**0.323**	0.079	0.072	0.116	0.087	0.067
B	0.063	0.088	0.074	**0.482**	0.087	0.085	**0.304**	0.124	0.087	0.084	0.082	0.068
C	0.077	0.067	**0.268**	0.123	0.068	0.155	0.108	0.13	0.097	0.107	0.108	0.066
D	0.079	0.098	0.095	0.083	0.517	0.089	0.117	0.075	0.15	0.116	0.164	0.094
E	0.127	**0.215**	**0.375**	0.115	0.114	0.102	0.111	0.153	0.087	0.071	**0.365**	0.064
F	0.128	**0.315**	**0.212**	0.156	0.159	0.164	0.107	0.096	0.094	0.086	0.102	0.082
G	0.108	0.18	0.133	0.166	0.125	0.09	0.168	0.45	0.26	0.111	**0.422**	**0.291**
H	0.094	0.098	0.152	0.16	0.102	0.172	0.112	0.122	0.122	0.121	0.129	0.081

C6	1	2	3	4	5	6	7	8	9	10	11	12
A	0.072	0.059	0.136	**0.456**	0.105	0.128	**0.108**	0.12	0.147	0.082	0.082	0.071
B	0.06	0.073	0.064	**0.539**	0.129	0.105	**0.316**	0.123	0.103	0.087	0.073	0.076
C	0.058	0.07	**0.11**	0.093	0.088	0.139	0.104	0.085	0.095	0.11	0.071	0.051
D	0.072	0.076	0.088	0.089	0.563	0.093	0.083	0.087	0.102	0.066	0.088	0.063
E	0.068	**0.084**	**0.09**	0.094	0.092	0.108	0.089	0.079	0.076	0.085	**0.145**	0.068
F	0.073	**0.092**	**0.15**	0.12	0.216	0.107	0.085	0.111	0.074	0.086	0.081	0.116
G	0.053	0.084	0.116	0.073	0.079	0.091	0.079	0.223	0.135	0.065	**0.149**	**0.066**
H	0.087	0.068	0.11	0.287	0.074	0.07	0.059	0.078	0.065	0.075	0.064	0.066

T7	1	2	3	4	5	6	7	8	9	10	11	12
A	0.076	0.148	0.096	0.086	0.068	0.083	0.077	0.129	0.102	**0.553**	0.075	0.133
B	0.064	0.073	0.08	0.073	0.072	0.074	0.07	0.077	0.133	0.159	0.1	**0.57**
C	0.067	0.074	0.102	**0.352**	0.074	0.07	0.126	0.103	0.113	0.174	0.068	0.13
D	**0.574**	**0.243**	0.135	**0.259**	0.108	**0.186**	0.107	**0.213**	0.17	0.08	0.08	0.068
E	0.067	0.068	0.076	0.07	0.091	0.124	**0.368**	0.115	0.133	0.119	0.067	0.09
F	0.066	0.065	0.07	0.084	0.121	0.15	0.155	0.193	0.115	0.087	0.107	0.067
G	**0.233**	0.083	0.106	0.108	0.096	0.144	0.123	0.108	0.186	**0.361**	0.112	0.059
H	0.06	0.091	**0.398**	0.073	0.122	**0.358**	0.092	0.072	**0.561**	**0.219**	0.076	0.074

C7	1	2	3	4	5	6	7	8	9	10	11	12
A	0.084	0.223	0.229	0.112	0.127	0.108	0.085	0.202	0.094	**0.626**	0.102	0.067
B	0.098	0.141	0.152	0.148	0.231	0.105	0.122	0.177	0.099	0.359	0.11	0.105
C	0.085	0.149	0.296	**0.158**	0.144	0.145	0.262	0.173	0.215	0.346	0.133	0.119
D	**0.686**	**0.438**	0.222	**0.48**	0.248	**0.462**	0.228	**0.472**	0.232	0.137	0.095	0.065
E	0.131	0.173	0.213	0.161	0.217	0.125	**0.446**	0.115	0.12	0.151	0.079	0.055
F	0.078	0.118	0.185	0.187	0.225	0.128	0.207	0.391	0.098	0.084	0.092	0.082
G	**0.58**	0.168	0.178	0.158	0.163	0.337	0.1	0.097	0.159	**0.438**	0.097	0.065
H	0.084	0.117	**0.628**	0.146	0.256	**0.608**	0.232	0.095	**0.349**	0.123	0.076	0.069

T8	1	2	3	4	5	6	7	8	9	10	11	12
A	0.078	0.07	0.102	0.085	0.112	0.134	0.099	0.101	0.123	0.094	0.073	0.086
B	0.073	0.174	0.076	0.197	0.188	0.187	0.112	0.122	**0.473**	0.173	0.098	0.055
C	0.101	0.089	**0.208**	**0.226**	0.187	**0.421**	**0.401**	**0.33**	**0.311**	**0.422**	0.128	0.07
D	**0.203**	0.133	**0.483**	**0.242**	**0.274**	**0.25**	**0.585**	**0.232**	**0.283**	0.164	0.102	0.083
E	0.063	0.084	**0.233**	**0.269**	**0.286**	**0.357**	**0.227**	**0.282**	0.136	0.175	0.14	0.073
F	0.062	0.102	**0.253**	**0.284**	**0.273**	**0.434**	0.095	0.098	0.128	0.101	0.056	0.066
G	0.062	0.121	**0.2**	**0.319**	0.169	**0.357**	**0.407**	0.113	0.077	0.068	0.055	0.069
H	0.062	0.082	0.082	0.087	0.121	0.136	0.101	0.074	0.069	0.168	0.077	0.07

C8	1	2	3	4	5	6	7	8	9	10	11	12
A	0.123	0.077	0.091	0.13	0.093	0.158	0.131	0.089	0.143	0.088	0.083	0.082
B	0.107	0.125	0.138	0.116	0.096	0.209	0.132	0.121	**0.123**	0.132	0.093	0.076
C	0.102	0.086	**0.094**	**0.138**	0.127	**0.776**	**0.373**	**0.262**	**0.197**	**0.489**	0.102	0.062
D	**0.25**	0.218	**0.441**	**0.317**	**0.213**	**0.202**	**0.635**	**0.173**	**0.315**	0.114	0.126	0.062
E	0.079	0.115	**0.121**	**0.164**	**0.198**	**0.371**	**0.166**	**0.303**	0.208	0.17	0.065	0.056
F	0.083	0.081	**0.094**	**0.119**	**0.227**	**0.65**	0.316	0.347	0.314	0.167	0.068	0.056
G	0.075	0.093	**0.082**	**0.339**	0.18	0.522	**0.472**	0.245	0.136	0.096	0.058	0.058
H	0.072	0.076	0.128	0.105	0.126	0.202	0.125	0.112	0.126	0.085	0.061	0.058

Note: T and C mean test and control plates, respectively. The fusion was carried out with the spleen from mouse 1. ELISA was performed as described in section 5.2.4. 143 positive supernatants, as shown in bold, were selected for rescreening with a competitive ELISA, as described in section 5.2.4.

Appendix 4 Screening hybridomas producing anti-chTim4SmAbs

Approximately 2,000 hybridomas (twenty 96-well plates) were screened by indirect ELISA.

T1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.132	0.143	0.069	0.074	0.138	**0.347**	0.097	0.208	0.078	0.142	0.058	0.052
B	0.095	0.109	0.11	0.135	0.074	0.072	**0.189**	0.194	0.127	**0.321**	0.134	0.057
C	0.093	0.18	0.087	0.068	0.068	0.065	0.065	0.06	0.09	0.07	0.058	0.054
D	0.078	0.077	0.071	0.081	0.071	0.066	0.258	0.132	0.059	0.225	0.204	*0.279*
E	0.073	0.081	0.113	0.077	0.094	0.062	0.063	0.087	0.1	0.077	0.056	0.135
F	0.094	0.09	0.076	0.066	0.074	0.06	0.066	0.072	0.061	0.105	0.055	0.065
G	0.091	0.097	**0.266**	0.08	0.076	0.068	**0.163**	0.065	0.133	0.151	0.073	0.055
H	0.063	0.063	0.074	0.167	0.284	**0.164**	0.071	0.202	0.082	0.089	0.064	0.132

T2	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.281**	0.087	0.083	**0.206**	0.084	0.07	0.068	0.066	0.074	0.082	0.095	0.139
B	0.083	0.119	0.092	0.079	0.146	0.077	0.074	0.069	0.066	0.08	0.059	0.057
C	0.126	0.069	0.07	0.079	0.125	0.061	0.062	0.059	0.06	0.059	**0.184**	0.054
D	0.062	0.062	0.071	0.08	**0.187**	0.063	0.061	0.057	0.063	0.059	0.056	0.059
E	0.056	0.257	0.124	0.088	0.061	0.068	0.059	0.06	0.059	0.062	0.055	0.078
F	0.066	0.072	0.083	0.059	0.116	0.058	**0.18**	0.077	0.106	0.059	0.126	0.105
G	0.144	0.066	**0.187**	0.093	0.083	0.135	0.062	0.072	0.066	0.078	0.071	0.061
H	0.066	0.065	0.054	0.056	0.055	0.064	0.072	0.087	0.062	0.306	0.063	0.073

T3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.115	0.088	0.063	0.07	0.076	0.068	0.062	0.059	0.129	0.063	0.057	0.056
B	0.053	0.064	0.113	0.064	0.061	**0.144**	0.069	0.1	0.058	**0.281**	0.059	0.06
C	**0.19**	0.062	0.147	0.058	0.162	0.066	0.065	0.106	0.065	0.102	0.064	0.054
D	**0.29**	0.09	0.122	0.06	0.16	0.061	**0.186**	0.061	0.06	0.056	0.055	0.06
E	**0.25**	**0.17**	0.082	0.06	0.095	0.062	0.061	0.061	0.1	0.056	0.082	**0.203**
F	0.062	0.06	**0.174**	0.058	0.068	0.057	0.133	0.059	0.063	0.107	0.088	0.122
G	**0.147**	**0.151**	0.107	**0.156**	0.099	0.085	0.059	0.062	0.064	**0.24**	0.108	0.11
H	0.106	0.057	**0.179**	0.06	0.118	0.062	0.099	0.065	0.056	0.059	**0.181**	0.054

T4	1	2	3	4	5	6	7	8	9	10	11	12
A	0.052	0.053	0.053	**0.191**	0.096	0.07	0.057	0.063	0.129	0.083	0.054	0.062
B	0.056	0.069	0.063	0.066	0.106	0.092	0.095	0.07	**0.217**	0.069	0.08	0.084
C	0.119	0.1	0.058	0.061	0.063	0.063	0.06	0.078	0.061	0.074	0.158	0.067
D	0.118	0.125	0.072	0.06	0.095	0.062	0.097	0.086	0.108	0.06	0.06	0.056
E	**0.235**	0.058	0.079	0.057	0.057	0.13	**0.185**	0.104	0.14	0.06	0.135	0.082
F	0.053	0.054	0.06	0.057	0.059	0.058	**0.244**	**0.218**	0.105	0.057	0.077	0.069
G	0.054	0.055	0.054	0.059	0.058	0.061	0.074	0.11	0.062	0.063	0.055	0.075
H	0.05	**0.284**	0.056	0.103	**0.164**	0.075	0.129	0.065	0.097	0.059	0.051	0.116

T5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.099	0.127	0.158	0.068	**0.201**	0.074	0.077	0.08	**0.213**	0.065	0.141	0.088
B	0.07	0.058	0.065	0.071	**0.233**	0.067	0.07	0.17	0.065	0.143	0.065	0.065
C	0.102	0.071	0.141	0.065	0.073	0.063	0.107	0.063	0.065	0.095	0.063	0.127
D	0.061	0.058	0.06	**0.172**	0.063	0.064	0.07	0.119	0.1	**0.216**	**0.171**	0.088
E	0.12	0.061	0.063	0.061	0.09	0.085	0.062	0.092	**0.185**	0.143	0.07	0.074
F	0.134	0.063	0.059	0.067	0.061	0.081	0.096	**0.229**	0.064	0.11	0.091	0.057
G	0.075	0.071	0.077	**0.215**	**0.182**	0.066	0.074	**0.304**	0.076	0.131	0.065	0.061
H	0.057	0.059	**0.185**	0.091	0.076	0.129	0.067	0.067	0.083	0.149	0.086	0.058

T6	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.308**	0.064	**0.286**	0.09	0.074	0.068	0.073	0.089	0.073	0.084	0.072	**0.21**
B	0.058	0.059	0.103	0.064	0.103	0.092	0.102	0.076	**0.247**	0.083	**0.183**	**0.196**
C	0.061	0.062	0.073	0.13	0.129	0.074	0.134	0.064	0.063	0.134	0.063	0.15
D	0.083	0.063	0.105	0.064	0.072	0.087	0.075	0.063	**0.163**	0.092	**0.383**	0.063
E	0.096	0.064	0.105	0.077	0.067	0.133	0.07	0.079	0.071	0.062	0.086	0.056
F	0.06	0.15	0.093	0.065	0.061	0.063	0.065	0.062	0.06	0.062	**0.266**	**0.245**
G	0.055	0.083	0.059	0.086	0.073	0.096	0.069	0.085	0.136	0.084	**0.189**	0.059
H	0.055	0.058	0.059	0.071	0.164	0.101	**0.358**	0.094	0.084	0.132	0.057	0.057

T7	1	2	3	4	5	6	7	8	9	10	11	12
A	0.139	**0.367**	0.071	0.077	0.116	0.065	0.081	0.067	0.064	**0.182**	0.109	0.063
B	0.085	0.059	0.068	0.115	0.128	0.067	0.142	0.064	0.075	**0.307**	0.062	**0.18**
C	0.081	0.084	0.062	**0.251**	0.065	0.062	0.071	0.063	0.061	0.061	0.061	0.057
D	**0.18**	0.059	**0.21**	0.092	0.086	0.064	0.063	0.059	0.06	0.059	0.059	0.058
E	0.055	0.071	0.078	0.151	0.066	0.091	0.067	0.059	0.163	0.075	0.056	0.055
F	0.054	0.06	**0.272**	0.063	0.06	0.133	**0.214**	0.1	0.082	**0.203**	0.061	0.061
G	**0.173**	0.06	0.063	0.059	0.061	0.076	0.126	0.067	0.06	0.06	0.057	0.095
H	0.081	0.057	0.064	0.152	0.077	**0.175**	0.066	0.063	**0.169**	0.152	0.06	0.053

T8	1	2	3	4	5	6	7	8	9	10	11	12
A	0.076	0.109	0.131	0.09	**0.304**	0.079	**0.307**	0.088	0.146	**0.291**	0.147	0.068
B	0.06	0.066	0.062	0.069	0.079	0.15	0.108	0.11	0.081	**0.284**	**0.183**	0.064
C	0.086	0.111	0.063	0.065	0.101	0.065	**0.207**	0.074	0.071	0.068	0.087	0.092
D	0.065	0.157	0.066	0.066	0.083	0.068	0.065	0.063	0.064	0.074	0.164	0.057
E	0.059	0.158	0.077	0.1	**0.256**	**0.346**	0.067	0.069	0.075	0.085	0.064	0.072
F	0.06	0.061	0.061	0.064	0.098	0.063	0.071	0.09	0.163	0.12	0.067	0.057
G	0.059	0.07	0.061	0.064	0.16	0.085	0.082	**0.192**	0.071	0.095	0.076	0.109
H	0.053	0.081	0.11	0.061	0.06	0.071	0.073	0.105	0.098	0.063	0.059	0.058

T9	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.221**	0.061	0.116	0.064	0.123	0.07	0.07	0.15	0.077	0.072	0.057	0.069
B	**0.289**	0.073	**0.318**	0.069	0.078	0.134	**0.173**	0.108	0.074	0.099	0.063	**0.381**
C	0.054	0.128	0.08	0.068	**0.322**	0.123	**0.177**	0.092	0.061	**0.166**	0.06	0.155
D	0.079	0.074	0.077	0.071	0.062	0.069	0.074	0.063	0.131	0.064	**0.169**	**0.279**
E	0.075	0.098	0.059	0.082	0.06	0.064	0.074	0.101	0.061	0.117	0.106	0.084
F	**0.24**	0.138	**0.323**	**0.177**	0.064	0.075	0.061	0.073	0.062	0.061	0.089	0.058
G	0.098	**0.192**	0.099	0.077	0.083	0.062	0.061	0.062	**0.241**	0.12	0.096	0.066
H	0.15	0.083	0.072	0.075	0.075	0.08	0.114	**0.197**	0.059	0.118	0.055	0.065

T10	1	2	3	4	5	6	7	8	9	10	11	12
A	0.141	0.058	0.059	0.078	0.057	0.061	0.11	0.059	0.072	0.198	0.057	0.057
B	0.059	0.068	0.064	0.061	0.079	0.057	0.054	0.057	0.072	**0.168**	0.054	0.054
C	0.142	0.104	0.055	0.07	0.056	**0.267**	0.061	0.056	0.131	0.056	0.12	0.055
D	0.056	0.086	0.088	0.056	0.056	**0.225**	0.057	0.062	0.099	0.097	0.053	0.054
E	0.057	0.055	0.057	0.058	0.057	0.146	0.156	0.061	**0.204**	0.06	0.053	0.057
F	0.14	0.061	0.056	**0.181**	0.059	**0.219**	0.065	0.056	0.067	0.068	0.052	0.056
G	0.059	0.053	0.053	**0.187**	0.093	0.064	0.086	0.054	0.071	0.055	0.052	0.058
H	0.121	0.058	**0.25**	0.073	0.07	0.069	0.056	0.123	0.102	0.109	0.055	0.058

T11	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.151	**0.309**	0.139	0.087	0.164	0.112	0.228	0.24	0.207	0.165	0.173	**0.407**
B	0.094	0.264	0.106	**0.308**	0.119	0.17	0.215	0.205	**0.323**	0.194	0.268	0.283
C	0.134	0.083	0.162	0.259	**0.379**	0.079	0.109	0.109	0.223	0.118	0.118	0.127
D	0.088	0.071	0.074	0.083	0.172	0.101	0.083	0.082	**0.31**	0.27	0.205	0.263
E	0.148	0.077	0.196	0.142	0.151	0.107	0.112	0.083	0.068	0.12	0.072	0.143
F	0.119	0.122	0.076	0.064	0.347	0.105	0.122	**0.335**	**0.352**	0.177	0.293	0.109
G	0.125	0.069	0.097	0.199	0.121	0.067	0.084	0.102	0.106	0.128	**0.448**	**0.412**
H	0.114	0.147	0.069	0.213	0.105	0.091	**0.47**	0.111	0.249	0.146	0.146	0.231

T12	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.169	0.186	0.112	0.187	**0.377**	0.107	0.181	0.176	0.248	0.209	0.282	**0.397**
B	**0.323**	0.124	0.198	0.127	0.132	0.22	0.071	0.092	0.323	0.088	0.246	0.21
C	**0.346**	0.107	0.077	0.141	0.105	0.121	0.099	**0.934**	0.243	0.232	0.151	0.24
D	0.202	0.251	0.143	0.204	0.066	0.065	0.094	0.174	0.089	0.117	0.066	**0.556**
E	0.196	0.218	0.11	0.166	0.073	0.295	0.069	0.102	0.069	0.066	0.094	**0.333**
F	0.23	0.268	0.267	0.138	0.107	0.103	0.195	0.154	0.06	0.282	0.066	0.253
G	0.083	0.123	0.079	0.206	0.1	**0.386**	0.123	**0.35**	0.132	0.074	0.162	0.219
H	0.265	**0.516**	0.109	0.087	0.081	0.199	0.221	0.164	0.191	0.087	0.075	0.193

T13	1	2	3	4	5	6	7	8	9	10	11	12
A	0.181	0.171	0.072	0.243	0.116	0.071	**0.457**	0.092	0.081	0.244	0.153	0.096
B	0.075	0.179	0.06	0.073	0.149	0.099	0.166	0.082	0.104	0.155	**0.516**	0.101
C	0.113	0.099	0.248	0.105	0.136	0.16	0.092	0.105	0.067	0.104	0.159	0.222
D	0.14	0.154	0.136	0.153	0.106	0.069	0.119	0.109	0.153	0.081	0.07	0.164
E	0.09	0.108	0.141	0.117	0.059	0.255	0.129	0.07	0.064	0.077	0.1	**0.353**
F	0.086	0.158	0.074	0.197	0.06	0.218	0.075	0.202	0.106	0.089	0.107	0.167
G	0.138	0.092	0.224	**0.339**	0.12	0.114	0.062	0.121	0.132	0.199	0.208	0.116
H	0.104	0.247	0.106	0.229	0.154	0.076	0.11	0.262	0.111	0.117	0.162	0.109

T14	1	2	3	4	5	6	7	8	9	10	11	12
A	0.144	0.26	0.108	0.228	0.136	0.137	0.119	0.122	0.092	**0.401**	0.108	**0.402**
B	0.2	0.183	0.147	0.172	0.114	0.273	0.221	0.101	0.249	0.281	0.186	0.17
C	0.165	0.221	0.144	0.097	0.146	0.249	0.145	0.089	0.7	0.236	0.114	0.239
D	0.225	0.107	0.21	0.156	0.163	0.288	0.298	0.105	0.137	0.138	0.146	0.28
E	0.129	0.088	0.161	0.198	0.161	0.22	0.121	0.095	**0.3**	0.155	0.148	0.229
F	0.168	0.117	0.257	**0.34**	0.203	0.183	**0.304**	0.105	0.115	0.139	0.114	0.149
G	0.231	0.164	0.078	0.161	0.117	0.141	0.172	0.143	0.123	**0.38**	**0.422**	0.242
H	0.135	0.169	**0.483**	0.129	0.132	0.168	0.118	0.216	0.249	**0.397**	0.133	**0.303**

T15	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.466**	0.159	0.146	0.105	0.089	0.168	0.23	**0.382**	**0.496**	0.327	0.323	0.139
B	0.149	0.14	**0.809**	0.106	0.204	0.181	0.195	0.387	0.226	0.18	0.254	0.179
C	0.196	0.356	0.178	0.11	**0.451**	0.31	**0.395**	0.218	**0.449**	0.1	0.193	**0.365**
D	0.249	0.31	0.242	**0.481**	0.225	0.123	0.212	0.28	0.263	0.179	0.135	0.169
E	0.256	0.189	0.299	0.072	0.331	0.086	0.073	0.147	0.106	0.127	0.411	0.28
F	0.178	0.31	0.113	0.145	0.217	0.076	0.108	0.362	0.196	0.21	**0.778**	0.268
G	0.141	0.134	0.269	0.114	0.225	**0.348**	**0.512**	0.185	0.158	0.095	**0.383**	0.27
H	0.263	0.225	0.116	0.226	0.19	**0.394**	0.28	0.384	0.099	0.227	0.253	0.305

T16	1	2	3	4	5	6	7	8	9	10	11	12
A	0.120	0.397	0.383	0.296	0.39	**0.519**	**0.415**	0.391	0.386	0.332	0.34	**0.502**
B	**0.498**	**0.437**	0.34	0.319	0.373	0.36	0.332	0.341	**0.411**	0.372	0.275	0.304
C	**0.446**	0.376	0.344	**0.581**	0.365	0.359	0.366	0.372	0.376	0.196	0.203	0.289
D	0.389	0.365	0.366	0.342	0.11	0.363	0.435	0.471	0.315	0.235	0.211	0.295
E	0.38	**0.4**	**0.439**	**0.434**	0.362	0.365	**1.008**	0.13	0.383	0.336	**0.576**	0.385
F	**0.515**	**0.687**	**0.431**	0.415	0.323	0.377	**1.072**	0.327	**0.468**	0.391	0.342	0.092
G	0.372	0.374	0.395	0.394	**0.426**	0.351	0.365	0.359	0.259	0.269	0.371	0.143
H	0.369	**0.459**	0.365	0.345	**0.408**	0.121	0.362	0.282	0.291	0.107	0.093	0.134

T17	1	2	3	4	5	6	7	8	9	10	11	12
A	0.228	0.266	0.19	**0.387**	0.35	0.368	0.271	0.084	0.357	0.361	0.351	**0.381**
B	0.285	**0.379**	0.241	0.347	0.252	0.369	0.278	0.346	0.387	0.364	0.23	**0.434**
C	0.29	**0.543**	0.258	0.219	**0.332**	0.307	0.302	0.353	**0.534**	0.371	0.325	**0.491**
D	**0.465**	0.233	0.302	0.341	0.352	**0.534**	0.333	**0.439**	**0.559**	0.366	**0.611**	**0.549**
E	0.353	0.321	0.339	**0.463**	0.368	0.37	0.339	0.285	0.37	0.258	0.388	0.338
F	0.191	0.172	0.241	0.164	**0.477**	0.342	0.349	**0.715**	**0.602**	0.263	0.28	0.264
G	**0.38**	**0.396**	0.327	0.215	0.368	**0.424**	0.335	0.36	**0.407**	0.367	0.217	0.353
H	0.135	0.272	**0.352**	0.21	0.128	0.159	0.263	0.197	**0.568**	**0.498**	0.124	0.356

T18	1	2	3	4	5	6	7	8	9	10	11	12
A	**0.362**	0.15	0.251	0.071	0.128	**0.46**	0.074	**0.331**	0.143	**0.354**	0.104	0.124
B	0.098	0.136	0.113	0.189	0.131	0.134	0.207	0.067	0.069	0.093	0.078	0.623
C	0.212	0.151	0.07	0.072	0.214	0.109	0.159	0.094	0.167	0.134	0.097	0.132
D	0.108	0.163	0.119	**0.449**	0.126	0.166	0.219	0.16	0.07	0.187	0.134	0.195
E	0.161	**0.351**	0.184	0.172	0.291	0.074	0.136	0.238	0.075	0.144	0.124	0.111
F	0.123	0.208	0.133	0.105	0.148	**0.427**	0.11	0.162	0.178	0.129	0.137	0.081
G	0.168	**0.292**	**0.747**	0.094	0.117	0.15	**0.326**	0.146	0.192	**0.356**	0.117	0.11
H	0.255	0.12	0.176	**0.55**	0.174	0.257	**0.342**	0.141	**0.311**	0.174	0.116	0.096

T19	1	2	3	4	5	6	7	8	9	10	11	12
A	0.169	0.101	0.309	0.113	0.267	0.105	0.105	**0.376**	0.156	0.536	0.208	**0.369**
B	0.174	0.091	0.074	0.099	0.154	0.118	0.121	**0.375**	0.082	0.098	0.127	0.159
C	0.244	0.21	0.096	0.094	0.091	0.093	0.142	**0.364**	0.078	0.212	**0.335**	0.131
D	0.153	0.097	0.175	0.152	**0.437**	**0.33**	0.143	0.12	**0.288**	0.219	0.135	0.236
E	0.078	0.08	0.156	0.075	0.15	**0.291**	**0.308**	0.205	0.225	0.142	0.205	**0.534**
F	0.089	0.135	0.206	0.166	0.247	0.172	0.1	**0.377**	0.219	0.267	0.169	0.135
G	**0.38**	0.108	0.125	0.113	0.134	**0.331**	**0.344**	0.108	0.099	0.114	0.142	0.111
H	**0.337**	0.252	0.282	0.122	0.248	**0.314**	0.22	0.245	0.141	0.391	0.171	0.133

T20	1	2	3	4	5	6	7	8	9	10	11	12
A	0.138	0.118	0.101	**0.284**	0.154	0.165	0.334	0.197	0.144	0.097	0.116	**0.277**
B	0.132	**0.225**	**0.269**	**0.225**	0.159	0.188	0.115	0.129	0.135	0.222	0.234	**0.268**
C	0.118	0.166	**0.296**	**0.295**	0.177	0.121	0.191	0.197	0.617	0.166	0.154	**0.275**
D	0.156	**0.326**	0.171	0.097	0.159	0.222	0.198	0.261	0.165	0.157	**0.22**	0.192
E	0.157	0.24	**0.237**	0.192	0.122	0.097	0.216	0.135	**0.29**	**0.31**	0.183	**0.265**
F	**1.255**	0.2	0.172	**0.221**	0.163	0.13	0.112	**0.391**	0.108	**0.343**	0.164	**0.377**
G	**0.364**	**0.545**	0.125	0.111	0.123	**0.548**	0.199	**1.206**	0.163	**0.317**	0.137	**0.349**
H	**0.243**	0.101	0.123	0.171	0.214	0.107	0.129	0.264	0.133	0.183	**0.243**	**0.331**

Note: T means test plate. Test plates 1-10 were a fusion with the spleen from mouse 1 and test plates 11-20 a fusion with the spleen from mouse 2. ELISA was performed as described in section 5.2.4. Based on the ELISA readings, 256 positive hybridomas, as shown in bold, were obtained and then grown up in 24-well plates.